Biomarkers of increased intestinal permeability in chickens

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Contents

Abstract

Enterocytes comprising the single layer of epithelial cells in the intestine are linked together by a series of proteins known as tight junction proteins (TJP). TJP absorb nutrients through selective permeability and act as a barrier between intestinal contents and blood. When the barrier function is compromised this is known as increased intestinal permeability (IP). The aim of the thesis was to investigate biomarkers of increased IP in chickens. Increased IP has been studied extensively in rats, pigs and humans. In contrast, this is a relatively a new area in chickens (Chapter 2). Based on published studies in rats and pigs, lipopolysaccharide (LPS), an endotoxin produced by Gram-negative bacteria, was selected as a method for increasing IP in chickens.

Three experiments were conducted utilizing LPS to increase IP in chickens (Chapter 3). These studies concluded that LPS failed to increase IP as measured by permeability of lactulose, rhamnose and mannitol sugars (LMR) and fluorescein isothiocyanate dextran (FITC-d) across the epithelial barrier. However, this work generated useful data and indicated for the first time that 90 minutes post-oral gavage was the optimal time-point for blood sampling. Since fasting for 19.5 hours was applied in the protocol of LPS administration (Chapter 3), a second experiment was conducted with fasting for 19.5 hours and dextran sodium sulphate ingestion along with the LMR sugars and FITC-d (Chapter 4). Additional biomarkers such as intestinal fatty acid binding protein, fecal antitrypsin inhibitor, diamine oxidase and d-lactate were also utilized. This paper concluded that 19.5 hours fasting increased IP that could be evaluated by LMR sugar and FITC-d methods. However, DSS ingestion did not show any IP increase in chickens. Since fasting is routinely applied in the chicken meat industry, fasting and its effects on IP were further investigated (Chapter 5). This study revealed that fasting for as little as 4.5 and 9 hours also increased IP in chickens. The study also confirmed that both methods (LMR sugars and FITC-d) could be utilized to evaluate increased IP.

Chapter 6 focused on delayed feeding at the time of chicken placement at the farm. However, this study revealed that delayed feeding did not alter IP in very young chickens. Finally, as short-term fasting also increased IP in older chickens, a further study was conducted to investigate whether mRNA expression of gut barrier associated genes was altered (Chapter 7). This study showed that mRNA expression of claudin-3 only was significantly different from control for 9 and 19.5 hours fasting.

This thesis has identified several biomarkers that could be utilized to evaluate increased IP in chickens. Some of these biomarkers such as fecal antitrypsin inhibitor, fatty acid binding protein, diamine oxidase and d-lactate could not detect IP changes induced by fasting in the current studies, they could potentially eliminate the need for oral gavage which is required in the FITC-d and LMR sugar tests. Nonetheless, this research project has shown that permeation of LMR sugars and FITC-d across the intestinal epithelium was able to demonstrate IP changes in chickens.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Signed Date: 24-06-2017

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List of publications by candidate included in the thesis

Gilani S, Howarth GS, Kitessa SM, Forder REA, Tran CD and Hughes RJ 2016. New biomarkers for intestinal permeability induced by lipopolysaccharide in chickens. Animal Production Science (in press).

Gilani S, Howarth GS, Kitessa SM, Tran CD, Forder REA and Hughes RJ 2016. Intestinal permeability induced by lipopolysaccharide and measured by lactulose, rhamnose and mannitol sugars in chickens. Animal, 1-6 (in press).

Gilani S, Howarth GS, Kitessa SM, Tran CD, Forder REA and Hughes RJ 2016: New biomarkers for intestinal permeability induced by dextran sodium sulphate and fasting in chickens Journal of Animal Physiology and Animal Nutrition (in press)

Gilani S, Howarth GS, Tran CD, Barekatain R, Kitessa SM, Forder REA and Hughes RJ 2017. Reduced fasting periods increase intestinal permeability in chickens. Journal of Animal Physiology and Animal Nutrition (in press).

Gilani S, Howarth GS, Tran CD, Kitessa SM, Barekatain R, Forder REA and Hughes RJ 2017. Effects of delayed feeding, sodium butyrate and glutamine on intestinal permeability in newly-hatched broiler chickens. Submitted to Journal of Animal Physiology and Animal Nutrition

Gilani S, Howarth GS, Nattrass G, Kitessa SM, Barekatain R, Forder REA, Tran CD, and Hughes RJ 2017. Gene expression and morphological changes in the intestinal mucosa

associated with increased permeability induced by short-term fasting in chickens. Submitted to Journal of Animal Physiology and Animal Nutrition.

Other presented work by candidate (not included in thesis)

Gilani S, Forder REA, Howarth GS, Hughes RJ, Kitessa SM and Tran CD. 2016. Biomarkers of increased intestinal permeability in chickens. Australian Poultry Science Symposium Sydney.

Gilani S, Howarth GS, Kitessa SM, Tran CD, Forder REA and Hughes RJ. 2016. New biomarkers for intestinal permeability induced by dextran sodium sulphate and fasting in chickens. World Poultry Science Association New Orleans USA.

Gilani S, Howarth GS, Kitessa SM, Tran CD, Forder REA and Hughes RJ. 2016. Lactulose, rhamnose and mannitol sugars test to assess increased intestinal permeability induced by lipopolysaccharide in chickens. World Poultry Congress Beijing China.

Gilani S, Howarth GS, Tran CD, Barekatain R, Kitessa SM, Forder REA and Hughes RJ. 2017. Reduced fasting periods increase intestinal permeability in chickens. Paper won first position at the Asia-Pacific regional level in the Alltech Young Scientist Competition (AYS) Kentucky USA. Also, presented at the ONE conference AYS Kentucky USA.

Barekatain R, **Gilani S**, Kitessa S M and Hughes RJ. 2017. Amino acids and intestinal barrier function: a case to be studied in reduced protein diets. Australian Poultry Science Symposium Sydney.

Thesis Structure – paper linkage

This thesis is presented as a series of six published or submitted journal articles. Due to the limited research in this particular area, a detailed literature review was conducted to investigate the models and biomarkers of increased intestinal permeability in chickens.

Paper 1 (Chapter 2): New biomarkers for intestinal permeability induced by lipopolysaccharide (LPS) in chickens, was a detailed literature review and revealed that LPS has been effectively utilized to increase IP in rats, mice, pigs and potentially could also be utilized in chickens. Furthermore, the review also investigated biomarkers of increased IP and concluded that lactulose, rhamnose and mannitol (LMR) sugars and fluorescein isothiocyanate dextran (FITC-d) could be utilized to evaluate increased IP in chickens. Additionally, other biomarkers, including intestinal fatty acid binding protein, zonula occludens (ZO-1), diamine oxidase (DAO), d-lactate and faecal antitrypsin inhibitor (AAT) were identified that could be utilized to evaluate increased IP.

Paper 2 (Chapter 3): Intestinal permeability induced by lipopolysaccharide and measured by lactulose, rhamnose and mannitol sugars in chicken, involved three independent experiments to investigate whether LPS (*Escherichia coli* 055:B5) could be effectively utilized to increase IP in chickens. This study investigated two different LPS doses of 0.5 and 1 mg/kg body weight with a time series to understand the LMR sugars and their passage from the intestine to the blood. Since birds in the first two studies were fasted over 19.5 hours, it was suggested that fasting might have an influence on IP increment and hence a third experiment with 1 mg/kg body weight LPS was conducted without fasting. Increased IP was evaluated with the LMR sugars, FITC-d and other potential biomarkers. The results showed that LPS did not increase IP. This third study demonstrated that the LPS effect was not masked by fasting in the earlier

study. In addition, these experiments identified that 90 minutes post oral gavage of LMR sugars was the optimal time to measure these sugars in blood.

Paper 3 (Chapter 4): New biomarkers for increased intestinal permeability induced by dextran sodium sulphate (DSS) and fasting in chickens. Following the inability for LPS to induce increased IP in broilers and a publication from other researchers regarding fasting and DSS ingestion in chickens, these two models were further investigated. In this study, which contrasted DSS and fasting, DSS showed no increment in IP, while fasting for 19.5 hours induced increased IP. This paper also revealed that LMR sugars and FITC-d could be utilized to evaluate increased IP in chickens. However, other potential biomarkers including faecal anti trypsin inhibitor, fatty acid binding protein, diamine oxidase, d-lactate and tight junction protein in the blood did not show any significant difference compared to control.

Paper 4 (Chapter 5): Reduced fasting periods increase intestinal permeability in chickens, focussed on shorter periods of fasting than Paper 3 above. Since fasting is unavoidable in the chicken meat industry in early placement and during the depopulation before slaughtering, the effect of short-term fasting on IP was further investigated. Additionally, one group of chickens was fed a glutamine supplemented diet (1%) to investigate the effect of glutamine prior to fasting. This study utilized FITC-d and LMR sugars and confirmed that both biomarkers could effectively be employed to evaluate increased IP. This study also revealed that as little as 4.5 and 9 hours of fasting increased IP. Glutamine supplementation failed to ameliorate increased IP in short-term fasting in chickens. Further studies are needed to investigate the optimal fasting time to avoid increased IP pre-slaughter. Once determined this could potentially reduce bacterial translocation in chicken intended for human consumption.

Paper 5 (Chapter 6): Effects of delayed feeding, sodium butyrate and glutamine on intestinal permeability in newly-hatched broiler chickens. Fasting is unavoidable in the early life of

chickens. Since previous experiments (Paper 3 and 4) investigated the effects of fasting in older birds (aged 21 - 38 days), this paper aimed to investigate whether delayed feeding at day 0 (during the placement from hatchery to farm), also increased IP. In addition, the study aimed to determine whether sodium butyrate and glutamine could ameliorate fasting-induced IP. The results showed that glutamine and sodium butyrate supplementation increased IP. Further research is required to determine their optimal doses. However, the results also showed that delayed feeding did not increase IP as measured by FITC-d on day 2, 4 and 7.

Paper 6 (Chapter 7): Gene expression and morphological changes in the intestinal mucosa associated with increased permeability induced by short-term fasting in chickens, investigated the molecular mechanisms of fasting-induced increased IP. This study was conducted using tissues from the previous study (Paper 4) to reduce the number of animals. The aim of the study was to determine changes in candidate genes assumed to be involved in intestinal barrier function. mRNA expression of the tight junction proteins (TJP) that link intestinal cells, for example zonula occludens (ZO-1, ZO-2), claudin-1, junctional adhesion molecules (JAM-2) and E-cadherin were not altered significantly. However, claudin-3 was significantly reduced for 9 and 19.5 hours fasting compared to control. This suggested that certain TJP may have been involved. However, non-significant reduction in claudin-3 expression following 4 hours fasting indicated a requirement for further research. Additionally, other genes, including glucagon like peptide-2 (GLP-2), heat shock protein (HSP-70), toll like receptor-4 (TLR-4), mucin (MUC) and cluster differentiation-36 (CD-36), which may have been involved in protection of gut cells, were not significantly altered. Fatty acid binding protein (FABP-6) expression reduced significantly, however, it was believed that it was possibly due to reduced bile acid production due to fasting.

Chapter 1: General introduction

Broiler meat production worldwide reached 114.9 million tonnes in 2015 (FAO 2016) and this industry has seen significant growth over the last two decades (FAO 2009, 2016). There are many reasons for the rapid expansion of the poultry meat business, for example quick return on the investment, high consumer demand and better feed conversion ratio (FCR). FCR is defined as the amount of feed required in kilograms (kg) to gain one kg of live weight and broilers need less than two kg of feed, compared with cattle which required approximately seven kg of feed (Fig 1).

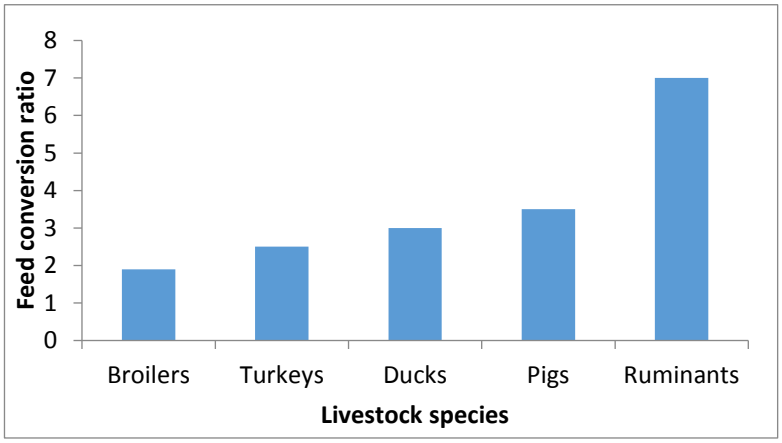

Figure 1: Global feed conversion ratios (kg feed/kg meat) of various livestock species (statistics courtesy of FAO food stat 2010)

According to the 2011 estimate of the Australian chicken meat industry (ACMI, 2011) the chicken meat industry grew over 36% in the world, and over 50% in Australia. Australian broiler meat production showed an upward trend that mirrored the global trend (Figure 2).

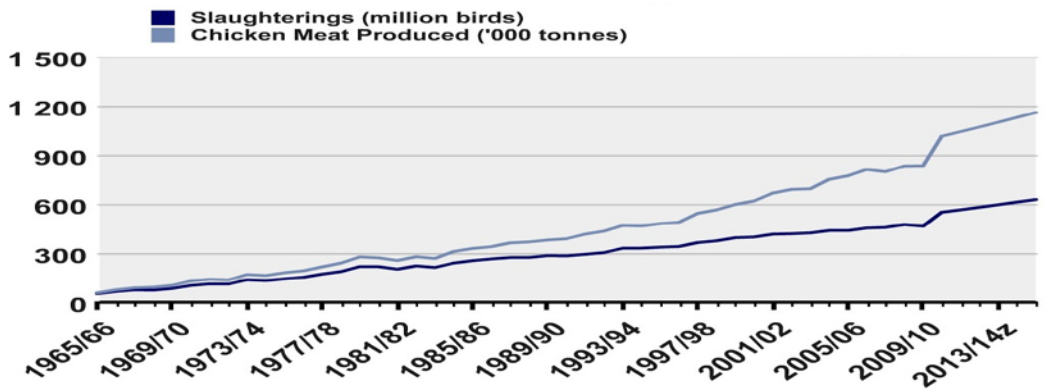

Figure 2: An upward trend of Australian chicken meat production for the last five decades (courtesy of ACMI 2011).

Similarly, FCR in the Australian broiler has improved significantly over the years as shown in Figure 3. The FCR can be increased when birds are challenged with various stressors in their early life. Stress is defined as 'any change in physiological and/or biochemical processes that results in deviation from the normal state of an organism and requires an adjustment to return to the normal state' (Keum et al., 2013).

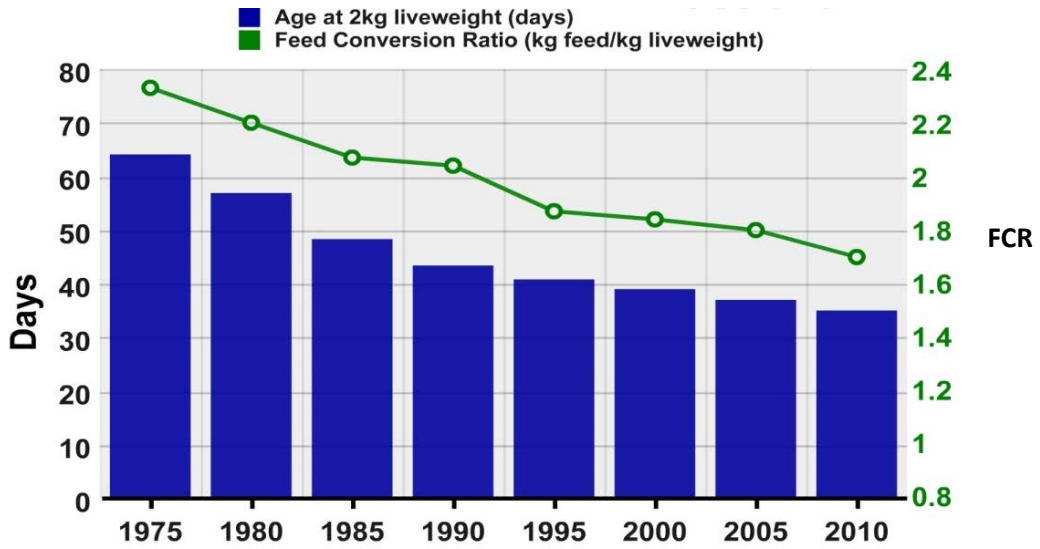

Figure 3: Improvement in the feed conversion ratio over the last 35 years (courtesy of ACMI 2011)

The small intestine has the most important role of food digestion and nutrient absorption across the epithelium (Leeson and Summers, 2001). Optimal gut functioning leads to improved FCR and healthy birds. However, stressors such as disease, pathogens and environment, can hinder both digestion and/or nutrient absorption, leading to reduced FCR and performance (Lochmiller and Deerenberg, 2000: Hanssen et al., 2004). A small change in FCR can lead to huge differences between meat yield and profit margin. The FCR in meat chicken has declined to almost 1.6:1 (Poultry Hub 2016). From a conservative estimate, almost one billion metric tonnes of feed was produced in 2017 globally (Alltech 2017). Out of this, almost 44% was used for poultry and even a slight change in FCR (0.01 – 0.1) would lead to extra consumption of feed (0.28 – 2.8 million tonnes) to achieve the same amount of chicken meat production.

The intestine consists of a single layer of epithelial cells, which not only absorbs nutrients but also keeps intestinal contents away from blood. However, due to stress or disease this structure may be compromised and lead to a condition known as leaky gut or increased intestinal permeability (IP) (Gilani et al., 2016). There is limited literature regarding increased IP in chickens and no literature regarding increased IP effects on FCR in chickens. In general, increased IP leads to increased bacterial translocation, activation of immune system and inflammatory response (Gilani et al., 2016). This shifts energy and nutrients away from production. Necrotic enteritis and coccidiosis are the two main diseases that cause significant losses in the poultry industry (Yegani and Korver, 2008). Necrotic enteritis has been shown to increase bacterial translocation to the liver (Liu et al., 2010) and to increase IP *in vitro* (Sun et al., 2015). Coccidiosis has also been suggested to increase IP (Rose and Long, 1969). Recently a model with high doses of coccidiosis vaccine increased IP in chickens (Chen et al., 2015). However, actual disease models were difficult to replicate in live animals due to live microorganisms being involved. Additionally, these models can also confound the results due to severe diseases and would not represent a mild intestinal challenge or stress that birds face

routinely. Furthermore, there is a currently little information regarding the models and biomarkers that could be utilized to study increased IP *in vivo* in chickens (Gilani et al., 2016).

Research aims

The main aim of this research project was to identify reliable biomarkers of increased intestinal permeability in chickens. Sugar ratio methods (lactulose, rhamnose and mannitol) have been utilized in human studies to investigate leaky gut. However, these have not been investigated in chickens. Additionally, there is limited literature of models that could be utilized to increase IP in chickens.

Accordingly, the aims of the research in chickens were to:

1. Establish an *in vivo* leaky gut model.
2. Evaluate IP changes in chickens utilising established biomarkers of increased IP (lactulose, rhamnose and mannitol sugars) in humans.
3. Develop new biomarkers of increased IP.
4. Investigate the effect of supplementation of glutamine and butyric acid on IP modulation in chickens.
5. Investigate the mechanism of fasting induced increased IP in chickens.

Chapter 2: Literature review – new biomarkers for intestinal permeability induced by lipopolysaccharide in chickens

Statement of Authorship

Title of Paper	New biomarkers for intestinal permeability induced by lipopolysaccharide in chickens
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
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Principal Author

Name of Principal Author (Candidate)	Syed Saad Akhtar Hussain Gilani		
Contribution to the Paper	Performed literature search, critically reviewed the literature, wrote the manuscript and acted as corresponding author		
Overall percentage (%)	75		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	22-02-17

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Gordon S Howarth		
Contribution to the Paper	Principal Supervisor, provided guidance on content and editing of manuscript		
Signature		Date	22/2/17

Name of Co-Author	Soressa M Kitessa		
Contribution to the Paper	Provided input about lipopolysaccharide and editing of manuscript		
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Name of Co-Author	Robert J Hughes		
Contribution to the Paper	Provided input of the poultry perspective and editing of manuscript		
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New biomarkers for intestinal permeability induced by lipopolysaccharide in chickens

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Abstract. Intestinal health is influenced by a complex set of variables involving the intestinal microbiota, mucosal immunity, digestion and absorption of nutrients, intestinal permeability (IP) and intestinal integrity. An increase in IP increases bacterial or toxin translocation, activates the immune system and affects health. IP in chickens is reviewed in three sections. First, intestinal structure and permeability are discussed briefly. Second, the use of lipopolysaccharide (LPS) as a tool to increase IP is discussed in detail. LPS, a glycolipid found in the outer coat of mostly Gram-negative bacteria, has been reported to increase IP in rats, mice and pigs. Although LPS has been used in chickens for inducing systemic inflammation, information regarding LPS effects on IP is limited. This review proposes that LPS could be used as a means to increase IP in chickens. The final section focuses on potential biomarkers to measure IP, proposing that the sugar-recovery method may be optimal for application in chickens.

Additional keywords: anti-trypsin inhibitor, fluorescein isothiocyanate dextran, intestinal fatty acid-binding protein, lactulose, leaky gut, models.

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Introduction

Intestinal health in animals is influenced by a complex set of variables involving intestinal structure, function (digestion and absorption; Meddings and Swain 2000), microbiota and immunity (Choct 2009). During the past couple of decades, there has been an increased interest in intestinal health, especially in relation to the role of resident microbes in health and disease. The small intestine is the primary site for nutrient absorption (Awad *et al.* 2008; Groschwitz and Hogan 2009) and consists of a single layer of epithelial cells, inter-dispersed with goblet cells, stem cells and entero-endocrine cells. The epithelial layer, covering finger-like projections (villi) as shown in Fig. 1, serves as a barrier between the harmful pathogens and sterile-blood circulation (Schokker 2012). At the luminal surface of the epithelial layer, there is a mucous layer that consists of mucin glycoproteins (Naughton *et al.* 2014). The mucous layer protects the epithelial layer; however, its composition and functions can be changed by environmental bacteria (Forder *et al.* 2007; Kitessa *et al.* 2014). Pathogens have to traverse the mucous layer before interacting and damaging the epithelial barrier (Naughton *et al.* 2014). During healthy physiologic function, pathogens cannot cross the barrier. However, for example, as a result of a disease or stress (Williams *et al.* 2013), intestinal barrier function is impaired, which can result in increased intestinal permeability (IP; Liu *et al.* 2010; Chen *et al.* 2015*a*), initiating an immune response (Schokker 2012) and, subsequently, inflammation.

An increase in IP in animals leads to compromised health and performance (Lochmiller and Deerenberg 2000; Hanssen *et al.* 2004) possibly due to increased bacterial or toxin translocation (Rosero *et al.* 2014; Ni *et al.* 2015), plasma leakage and protein wastage (Colman and Rubin 2014; Parambeth *et al.* 2015), with subsequent impaired growth and performance thus resulting in economic loss (Zuidhof *et al.* 2014). Antibiotics as growth promoters have been used in chicken feed to ameliorate enteric inflammation. Their usage as growth promoters has been banned in the European Union. Other countries, such as Australia and the USA, are now also working to reduce the loads of these antibiotics in animal feed (Laxminarayan *et al.* 2015). This further highlights the importance of studying IP in chickens (Vicuña *et al.* 2015). Although considerable studies have been conducted on intestinal diseases and increased IP in humans

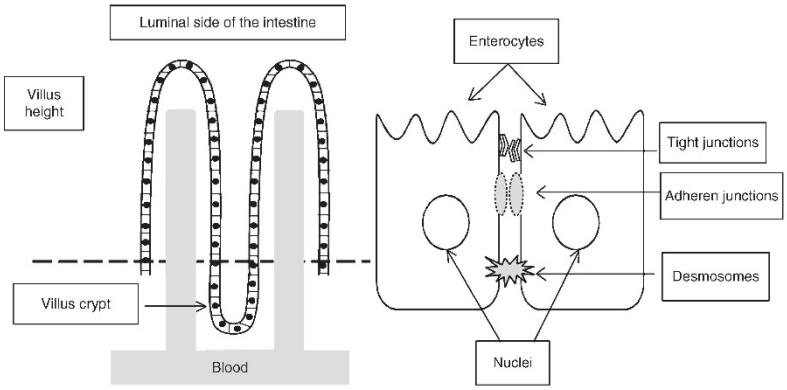

Fig. 1. Simplistic view of the intestinal villi and enterocytes with tight junctions (TJs), adherens junctions (AJs) and desmosomes. TJs regulate paracellular permeability. AJs and desmosomes (condensed plaque) link cells mechanically.

(Colman and Rubin 2014; Ni *et al.* 2015; Niewold 2015; Piton *et al.* 2015), pigs (Zhu *et al.* 2015) rats and mice (Williams *et al.* 2013; Ruan *et al.* 2014), this is a relatively new and developing area of research in chickens. Establishing a successful model of increased IP may help understand the mechanism of impaired intestinal barrier function and could lead to the development of reliable biomarkers of intestinal health and, subsequently, improve animal welfare. This review identifies knowledge gaps for increasing permeability as well as evaluating intestinal barrier function in chickens. The first part of the review introduces the morphology of the tight junctions and its relation to increased IP, followed by the potential use of lipopolysaccharide (LPS) as a model to increase IP; and finally, the potential of different biomarkers of IP is reviewed.

Tight junctions

The structural architecture and function of epithelial cells have been well described by Powell (1981) and Uni *et al.* (1998). Briefly, epithelial cells are connected tightly through a protein network consisting of desmosomes, adherens junction proteins and tight junction (TJ) proteins (Fig. 1). Adherens junction proteins, also known as zonula adherens, are associated with cell-to-cell contact. Adherens and desmosomes (condensed plaque-like structure) link cells mechanically. TJ, found between the tightly packed epithelial cells, has selective permeability for ions and nutrients. TJ is made up of integral membrane proteins, which are classified further into occludens, claudins and jams. Occludens and jams are involved in paracellular permeability (Groschwitz and Hogan 2009). Claudins are connected to the actin cytoskeleton via zonula occludens (ZO) and have a fundamental role in TJ formation (Ni *et al.* 2015).

TJs have been studied in depth in various species other than chickens. However, TJs have been studied in chickens by demonstrating that the expression of TJ proteins can be found in chicken embryos and immediately after hatching (Kimura *et al.* 1996; Karcher and Applegate 2008; Ozden *et al.* 2010). The expression showed its peak at 2–3 days post hatch. Furthermore, other studies have shown that TJ protein expression in chicken is present in later stages of life (Wang *et al.* 2014; Li *et al.* 2015).

Intestinal permeability in chickens

Intestinal permeability refers to the barrier properties of the intestinal mucosa that prevent harmful substances from penetrating the mucosa and entering the systemic circulation (Bjarnason *et al.* 1995; Hollander 1999). Probes used to measure intestinal permeability are passively absorbed water-soluble compounds that measure TJ intactness (Hollander 1999). Gut permeability can be assessed in cell culture by measuring trans-epithelial electrical resistance (TEER; Liu *et al.* 2013), in rodent intestine *in vitro* by utilising Ussing chambers (Neirinckx *et al.* 2011) or in animal models using ^{51}Cr-EDTA (Frias *et al.* 2004; Escala *et al.* 2006) and in chickens *in vitro* by Ussing chambers (Ruhnke *et al.* 2013; Ghareeb *et al.* 2015). Intestinal permeability in humans is conventionally assessed non-invasively *in vivo* by measuring urinary excretion of non-metabolised orally administered test substances (non-metabolised water-soluble probes); however, results are influenced by a change in pre-mucosal and post-mucosal factors (Bjarnason *et al.*1995). To remove confounding factors such as intestinal transit, completeness of urine collection and blood flow, a small and a large probe are conventionally given together, with the results expressed as a ratio between the large (e.g. lactulose) and small (e.g. mannitol or rhamnose) probes (Hollander. 1999). This led to the formulation of the principle of differential urinary excretion of test substances, which provides a surrogate marker of intestinal permeability.

There is limited literature investigating IP in chickens *in vivo*; however, factors that influence IP are well studied in other species, these include food allergy, alcohol and stress (Groschwitz and Hogan 2009) in humans. Increased IP in humans has been replicated using rat and mouse models, such

as the administration of the chemotherapy drugs methotrexate and 5-fluorouracil (Prisciandaro *et al.* 2011), radiation (Chun *et al.* 1997; Hang *et al.* 2007), environmental stressors (Meddings and Swain 2000) and consuming high-fat diets (Stenman *et al.* 2013; Hamilton *et al.* 2015). None of these stimuli has been applied to chickens *in vivo*, to provide a useful model of increased permeability. However, deoxinivalenol as a fungal toxin to increase IP in chickens has been developed (Ghareeb *et al.* 2015) for Ussing-chamber technique.

Gastrointestinal diseases in poultry, such as coccidiosis and necrotic enteritis, can lead to increased economic losses and reduced animal wellbeing (Williams 2005; Yegani and Korver 2008). Reviews in avian diseases have suggested that intestinal permeability might increase in coccidiosis (Williams 2005; Chapman 2014). This was actually tested in one study where the administration of *Eimeria* resulted in an increased IP, measured by its translocation (Rose and Long 1969). A recent study utilised higher doses of a coccidiosis vaccine to increase IP as measured by bacterial-toxin translocation (Chen *et al.* 2015*a*). This suggested that increased permeability precedes the development of diseases and, therefore, it is essential to establish a model of increased intestinal permeability in chickens to better understand the role of reversing intestinal permeability on disease development.

Some disease models, such as necrotic enteritis model, have been developed in chickens (Liu *et al.* 2010; Murugesan *et al.* 2014; Awad *et al.* 2015). There is very little known about necrotic enteritis and its relationship with increased IP, as clinical stages are too severe and subclinical stages can go unnoticed (Williams 2005). Just recently, feed restriction, orally administered dextran sodium sulfate and rye-based diets have been used to increase IP in chickens measured by fluorescein isothiocyanate dextran (FITC-d) method (Kuttappan *et al.* 2015; Vicuña *et al.* 2015). These studies suggested that since feed restriction and dextran sodium sulfate have caused enteric inflammation in rats and mice, similar mechanisms might have led to increased IP in chickens. Similarly, rye-based diets were suggested to have caused intestinal inflammation due to its non-starch polysaccharide content. However, the exact mechanism in chickens requires further confirmatory experiments. However, LPS has been used to induce systemic inflammation, resulting in increased IP in mice, rats and pigs (discussed in detail in the section *Lipopolysaccharide (LPS)* below, and shown in Tables 1–4). Although LPS has been used to induce systemic inflammation in chickens, knowledge regarding its effects on IP is limited and is discussed in the following section.

Lipopolysaccharide (LPS)

The structure and origin of LPS has been well documented (Tran and Whitfield 2009). Briefly, Gram-negative bacteria have an outer layer made up of a lipid bilayer. The inner side of the lipid bilayer is composed of glycerophospholipids and the outer layer is made up of an LPS, known as endotoxin, which has also been used to induce systemic inflammation and to increase IP in animals. Detailed studies of *in vitro* cell lines, rats, mice, pigs and chickens are reviewed below and summarised in Tables 1–4.

LPS increased intestinal permeability in in vitro *studies*

In vitro studies have shown that LPS increases IP in mouse cells (Han *et al.* 2013), in human intestinal cells (Nébot-Vivinus *et al.* 2014) and disrupts TJ of cell cultures (Lei *et al.* 2014; Chen *et al.* 2015*b*; Table 1). Briefly, an LPS dose of 1 ng/mL to 1 μg/mL increased IP as measured by TEER, FITC-d transport and ZO-1 expression. FITC-d is 3–5 kDa size sugar, which can pass through TJ only due to increased IP. *In vitro* studies allow investigation of responses at the cellular level and can be very efficient. The results of *in vitro* studies of mouse cells (Han *et al.* 2013) were comparable to those of *in vivo* studies (Chow *et al.* 1999) in which LPS increased IP, showing the importance of *in vitro* studies. Cell lines for chicken intestine have been developed recently (Dong *et al.* 2006). However, no published literature has been found on different agents on increasing IP in chicken cell lines. Cell lines have one limitation, namely, that animal or human epithelium consists of more than one type of cells, compared with only enterocytes in the cell lines. Additionally, there is mucous and luminal content in healthy intestine compared with unstirred water and no mucous in the cell lines (Lea 2015).

LPS mechanism for increasing intestinal permeability

Studies in rats and mice have aided our understanding of the mechanism of LPS action for inducing inflammation and increasing IP (Table 2). LPS is recognised by the LPS-binding protein and cluster-differentiation (CD14) proteins on macrophages. These proteins activate toll-like receptors (TLR-2 and TLR-4; Hornef *et al.* 2003; Neal *et al.* 2006; Chaussé *et al.* 2011; Mani *et al.* 2012). It is now known that TLR-4 and CD14 are responsible for immune activation (Mani *et al.* 2012; Williams *et al.* 2013). This, in turn, activates the nuclear factor NF-kβ, a protein that controls gene transcription of pro-inflammatory cytokines (as activated by LPS at 1 μg/mL in *in vitro* study) through TLR4 receptors (Chow *et al.* 1999). The pro-inflammatory cytokines including tumour necrosis factor-α (TNF-α) and interleukins IL-1 and IL-6 promote an inflammatory response (also known as systemic inflammation) and increase intestinal IP (Mani *et al.* 2012; Tan *et al.* 2014). This was established when LPS did not change IP in mice deficient in TNF-α and NF-kβ2. Additionally, it was also suggested that cellular processes occurred rapidly in the intestine. Senescent cells became apoptotic (and shed off) and new cells proliferated, differentiated, matured and migrated to the surface. If intact cells were not formed due to stress (LPS), then TJs were also disrupted leading to increased IP (Williams *et al.* 2013). In summary, pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 produced in response to stress or immune activation increase IP.

LPS increased intestinal permeability in mice and rats

A study in mice showed that repeated LPS injections (*E.coli* O111:B4, 0.1 mg/kg bodyweight (BW), intra-peritoneal five times) increased expression of *TLR4* and *CD14* genes. *TLR4* and *CD14* genes are responsible for recognising LPS and inducing an immune response. Increased expression of these genes has been linked to an increase in IP (Guo *et al.* 2013). Williams *et al.* (2013) demonstrated that administration of

Table 1. Summary of *in vitro* studies used for understanding the lipopolysaccharide (LPS) mechanism and for increasing intestinal permeability (IP)

FITC, fluorescein isothiocyanate; n.a., not available; TEER, trans-epithelial electrical resistance

Author	Repeat	LPS dose	Cell-line species	Bacteria	Effects measured	Other effects and information
Chow *et al.* 1999	n.a.	1 μg/mL	Human embryonic kidney cell line	n.a.	Systemic inflammation. Toll-like receptor activation (TLR4). Increased gene expression of nuclear factor kappa B and cluster differentiation CD14.	Activation of the immune system. Measured signalling pathway of LPS but not increased IP directly.
Guo *et al.* 2013	n.a.	10 ng/mL	Human epithelial colorectal adenocarcinoma cells (Caco-2)	*E.coli* O111:B4	Systemic inflammation. TLR and CD14 cell expression increased, showing increased gut permeability.	Increased IP significantly in cell line that was measured by TEER by VM. Very good study for understanding the mechanism of increased IP.
Han *et al.* 2013	n.a.	n.a.	Human colon cells	n.a.	IP increased significantly, measured by TEER by Voltohmmeter (VM)	
Nébot-Vivinus *et al.* 2014	n.a.	10 ng/mL	Caco-2	n.a.	LPS increased colonic permeability perfusion of a faecal supernatant significantly, measured by Transwell filter (TF).	Lactibiane yolerance (probiotic) mitigated the effects of LPS.
Chen *et al.* 2015*b*	n.a.	1–10 ng/mL	Caco-2	Mix of *E.coli* O111: B4 and O55:B5	IP measured by TEER and FITC 40 flux, but significantly increased by TF.	1, 25-Dihydroxyvitamin D3 mitigated the effects of LPS.
Lei *et al.* 2014	n.a.	1 ng/mL	Caco-2	*E.coli* 0111:B4	IP measured by TEER flux by VM, expression of ZO1, occludens and Claudin 1, phospho-nuclear factor kappa B.	These *in vitro* studies have two limitations. First, these have been conducted in human cell lines of colon epithelium. Second, cell lines lack live animal responses in general.

a single LPS injection (*E.coli* O111:B4, intra-peritoneal, 10 mg/kg BW) was an excellent model of intestinal permeability in mice, as measured by FITC-d (Table 2).

The studies utilising lipopolysaccharide in rats for intestinal inflammation and increased IP are discussed below. In an earlier study in rats, a single intra-peritoneal injection of LPS (*E.coli* O111:B4, 20 mg/kg BW) increased expression of nitric oxide synthase, which could lead to lipid peroxidation and villus sloughing (Mercer *et al.* 1996). Similarly, a single LPS injection in rats (*E.coli* O111:B4, intra-peritoneal, 20 mg/kg BW) was effective at inducing inflammation by increasing TNF-α, IL-1β, NF-kβ and nitric oxide and inducing villus injury (Zhang *et al.* 2011). Although TNF-α, IL-1, NF-kβ are known to increase IP as discussed above, IP was not measured in that study. On the basis of previous studies in mice (Guo *et al.* 2013), a single LPS injection (*E.coli* O55:B5, intra-peritoneal, 5 mg/kg BW) was used as a model to increase IP in rats measured by an everted gut-sac method (Yue *et al.* 2013). A recent study in rats showed that a single LPS injection (intra-peritoneal, 1 mg/kg BW) caused villus atrophy, decreased expression of ZO proteins, increased diamine oxidase (DAO) activity and increased IP, measured by the lactulose and mannitol sugar test (Ruan *et al.* 2014). Earlier studies in rats used high doses of LPS (20 mg/kg BW); however, recent studies confirmed that a single dose of LPS (1 mg/kg BW) was sufficient to increase IP in rats (Table 2).

LPS increased intestinal permeability in pigs

Lipopolysaccharide infusion (*E.coli* O111:B4, intravenous, 7.5 μg/kg BW) has been shown to damage ZO-1 proteins (Klunker *et al.* 2013), suggesting a role in compromising IP (ZO-1 is an integral component of TJ; Table 3). LPS (*E.coli* K-235, intravenous, 5 μg/kg BW) has been shown to significantly decrease IP as measured by TEER in Yorkshire pigs, whereas the administration of LPS to Meishans pigs did not change IP, suggesting that some breeds of a particular species as well as different species may respond differently to LPS. Both studies were conducted in the same experiment. Yorkshire pigs were heavier than Meishans pigs; however, LPS was administered on BW, suggesting that different BW should not change results (Albin *et al.* 2007). The contrasting results in these studies could have been due to the difference in LPS dose used (5 vs 7.5 μg/kg BW). LPS (*E.coli* O111:B4, intravenous, 250 μg/kg BW) increased IP in pigs measured by labelled EDTA and urea in intestinal contents (Fink *et al.* 1991). Additionally, LPS (*E.coli* 055: B5, intra-peritoneal at 100 μg/kg BW) increased IP measured by DAO in blood (Liu *et al.* 2012). In a recent

Table 2. Summary of lipopolysaccharide (LPS) studies for increasing intestinal permeability in mice and rats
BW, bodyweight; IP, intestinal permeability; TEER, trans-epithelial electrical resistance

Author	Animals, sex and age	Repeat	LPS route and dose	Bacteria	Effects measured	Other effects
Williams *et al.* 2013	Wild mice, not available and 63 days	No	Intra-peritoneal, 10 mg/kg BW	*E.coli* O111:B4	IP increased significantly measured by fluorescein-isothiocyanate-conjugated dextran (FD4) through plasma.	Intestinal morphology. Villus apoptosis. Less invasive biomarker used.
Guo *et al.* 2013	Mice, male and 63 days	Five times	Intra-peritoneal, 0.1 mg/kg BW	*E.coli* O111:B4	IP increased significantly measured by water absorption capacity and TEER.	Intestinal perfusion method was used that is more invasive than FD4 method.
Mercer *et al.* 1996	Rats, female Sprague Dawley and age not available	No	Intra-peritoneal, 20 mg/kg BW	*E.coli* 0111:B4	Intestinal morphology. Villi affected and sloughed off.	i-NOS (nitric oxide synthase) expression indicated that it may have caused intestinal damage.
Ruan *et al.* 2014	Rats, male Sprague Dawley and 21–22 days	No	Intra-peritoneal, 1 mg/kg BW	Not available	IP increased significantly and measured by Zonula and occludens tight junction protein. Intestinal morphology measured by villus height.	Lactulose and mannitol measured in urine as a measure of increased IP. Chlorogenic acid helped improve gut permeability measured by western blot analysis for occludens and zonula occludens-1 (ZO-1).
Yue *et al.* 2013	Rats, male Sprague Dawley and age not available	No	Intra-peritoneal, 5 mg/kg BW	*E.coli* O55: B5	IP increased significantly measured by everted gut-sac procedure and labelled *E.coli*	Histology with haematoxylin and eosin staining.

experiment, LPS (*E.coli* O55:B5, intra-peritoneal 200 µg/kg BW) downregulated mRNA expression of ZO-1 TJ, suggesting that LPS increased IP (Zhu *et al.* 2015). In summary, LPS administered as an intravenous infusion of 7.5 µg/kg BW or as an intra-peritoneal injection at a dose of 100 µg/kg BW is sufficient to increase IP in pigs (Table 3).

LPS increased IP in chickens

Published research has shown that administration of LPS in chickens has been readily used as a model of systemic inflammation (Xie *et al.* 2000; Bowen *et al.* 2009; De Boever *et al.* 2009; Hu *et al.* 2011; Tan *et al.* 2014). However, very few studies have investigated the effects of LPS on IP. Here, we discuss the potential use of LPS administration as a model of IP.

Intracellular adhesion molecules are considered as binding agents between cells and regulate infiltration of the cytokines. Intracellular adhesion molecules were significantly affected by LPS treatment (*E.coli* O55:B5, oral route, 250 µg/kg BW) due to intestinal inflammation. Additionally, D-lactate and DAO were increased (Wu *et al.* 2013). Both of these markers have been used in mammals as an indicator of increased IP (discussed in section *Different biomarkers for evaluating increased intestinal permeability*). Repeated LPS treatment (*E.coli* O55:B5, intra-peritoneal, 1 mg/kg BW) also reduced mRNA expression of ZO-2 and occludens proteins, which are components of enterocyte TJ (Wang *et al.* 2014). Recently, a similar study with a lower dose of LPS (*E.coli* O55:B5, intra-peritoneal, 0.5 mg/kg BW) resulted in a higher DAO and a reduced expression of ZO-2 (Li *et al.* 2015). This suggests that LPS can also damage intestinal TJ proteins in chickens. In summary, an intra-peritoneal LPS treatment at a dose of 0.5–1 mg/kg BW, repeated twice, may increase IP in chickens (Table 4). Interestingly, Arbor acres broilers were used in all three studies, and further research is required in other breeds. As mentioned above in section *LPS increased intestinal permeability in pigs*, LPS may behave differently in a particular breed of a species. In contrast to the above studies, an *in vitro* study of chicken jejunum showed that the presence of either *S. enteritidis* (3×10^9 bacteria) or LPS isolated from *S. enteritidis* L6011 (20 mg/L) reduced intestinal ion permeability measured by TEER in a Ussing chamber (Awad *et al.* 2012). This suggests that LPS may act differently in chickens, as they are exposed to the LPS through faecal ingestion and continuous exposure may immunise chickens.

Different biomarkers for evaluating increased IP

Different biomarkers have been developed to assess intestinal health, including the intestinal microbiota and morphology (Stanley *et al.* 2012). However, very few studies have

Table 3. Summary of lipopolysaccharide (LPS) studies increasing intestinal permeability (IP) in pigs
BW, bodyweight; TEER, trans-epithelial electrical resistance

Author	Animals, sex and age	Repeat	LPS route and dose	Bacteria	Effects measured	Other effects
Klunker *et al.* 2013	Barrows pigs, castrated male and 37 days	Infused for 1 h once	Intravenous, 7.5 µg/kg BW	*E.coli* O111:B4	IP increment significantly measured by diminished zonula occludens (ZO) protein by immunoblotting. Intestinal inflammation. Crept depth did not change.	Crypt depth was associated with the segments of the intestine.
Albin *et al.* 2007	Yorkshire and Meishan pigs, female and 42–56 days	Once	Intravenous, 5 µg/kg BW	*E.coli* K-235	IP decreased significantly measured by reduced TEER in Ussing chamber in Yorkshire and non-significant difference in Meishans pigs.	Nutrient transport of glucose, arginine and glycylsarcosine was increased in LPS-treated group. Two different breeds had different BWs.
Zhu *et al.* 2015	Cross bred pigs, barrows and 14 days	Once	Intra-peritoneal, 200 µg/kg BW	*E.coli* O55:B5	IP increased significantly measured by downregulation of ZO-1 mRNA and increased endotoxins in plasma.	Increased malonaldehyde in plasma and also diarrhoea. Using endotoxins in plasma with LPS treatment might not be a conclusive biomarker.
Fink *et al.* 1991	Yorkshire mix bred pigs, male and age not available (10–13 kg BW)	Once	Intravenous, 250 µg/kg BW	*E.coli* O111:B4	IP significantly increased measured by increased leakage of labelled ^{51}Cr-EDTA and urea.	
Liu *et al.* 2012	cross bred Duroc × Large White × Landrace pigs, male and 21 days	No	Intra-peritoneal, 100 µg/kg BW	*E.coli* 055: B5	IP significantly increased measured by increased plasma diamine oxidase activity. Also decreased occludin and claudin-1 gene expression significantly.	It would have been interesting to compare sugar test in the above LPS-treated pig studies.

discussed IP in chickens. Additionally, there is a dearth of knowledge of different biomarkers available to evaluate increased IP. Different biomarkers used to evaluate IP, their mechanism, relevance to chickens and advantage(s) or disadvantage(s) of each biomarker, are discussed below (Table 5, Fig. 2).

Lactulose, L-rhamnose and mannitol sugar test

Lactulose (L), L-rhamnose (R) and mannitol (M) are non-digestible and non-metabolisable carbohydrates (or sugars). These sugars have been used to assess increased IP in humans, as reviewed by Tooley *et al.* (2009). L has a higher molecular weight (342 Dalton (D)) than have R (164 D) and M (182 D). In healthy animals, L passes through the stomach and small intestine undigested. If there is damage to the intestinal barrier, then it can pass through the TJ (known as the paracellular pathway) in high quantity and enter the blood stream, after which it is processed by the kidney and excreted from the body via urine (excreta in the case of chickens). Small molecular sugars (R and M) are absorbed through the intestinal epithelium by simple diffusion, known as the transcellular pathway (Bjarnason *et al.* 1995; Cox *et al.* 1999; Hollander 1999; Fig. 2). By measuring the ratios of these sugars through urine or blood, IP can be assessed.

A review by Jeurissen *et al.* (2002) suggested that using the LMR sugar method in chickens for measuring IP was impossible, since this method was validated in urine samples only and chicken void excreta. Later, a positive correlation was found between these sugars recovered in urine and blood samples in dogs (Sørensen *et al.* 1997), humans (Cox *et al.* 1999), rabbits and mice (Katouzian *et al.* 2005), suggesting that this technique could be used in serum or plasma as well as in urine. This further suggests that the LMR sugar test can be performed in chicken blood (plasma or serum). However, there has not been any published research on the recovery of these sugars in chicken blood and, hence, optimisation of this test in chickens is yet to be determined.

Fluorescein isothiocyanate dextran sugar test (FITC-d)

Fluorescein isothiocyanate dextran (FITC-d) is a fluorescein-labelled sugar similar to lactulose, but has a greater molecular size (3000–5000 D) than does L (342 D). Since FITC-d is larger in molecular size, it does not normally pass through TJ in high quantity unless the TJ are damaged (Tan *et al.* 2015). FITC-d has been used as a biomarker of increased IP in mice (Yuh *et al.*

Table 4. Summary of lipopolysaccharide (LPS) studies increasing intestinal permeability (IP) in chickens
DAO, diamine oxidase; FITC-d, fluorescein isothiocyanate dextran; ICAM, intracellular adhesion molecules; IFN, interferon; IL, interleukin; LMR, lactulose–L-rhamnose–mannitol; TNF, tumour necrosis factor-α; n.a., not available

Author	Animals, sex and age	Repeat	LPS route and dose	Bacteria	Effects measured	Other effects
Wu *et al.* 2013	Arbor acres broilers, sex not available and 16 days	Three times	Oral, 250 μg/kg BW	*E.coli* O55:B5	IP increased significantly. Measured by s-ICAM mucosal concentration, DAO and D-lactate. Systemic inflammation. IL and TNF were higher in LPS.	DAO and D-lactate are biomarkers of increased IP but not primary biomarkers compared with FITC-d or LMR sugars.
Wang *et al.* 2014	Arbor acres broilers, sex not available and 19 days	Two times	Intra-peritoneal, 1 mg/kg BW	*E.coli* O55:B5	IP increased significantly. Zonula-2 and occludens protein mRNA downregulated. Systemic inflammation measured by increased cytokines IL-1β, IFN-γ.	Protein expression maybe good indicator for increased IP but that is not an *in vivo* technique compared with sugars technique.
Li *et al.* 2015	Arbor acres broilers, male, 16,19 and 20 days	Three times	Intra-peritoneal, 10.5 mg/kg BW	*E.coli* O55:B5	IP increased significantly. Zonula-2 protein mRNA downregulated while occludens upregulated. DAO levels also increased.	Same as above two comments.
Awad *et al.* 2012	Layers and broilers, male and female and 6–16 weeks	n.a.	*In vitro* sections of chicken intestines, 20 mg/L	*S.enteritidis*	Ion permeability in gut was reduced by *S.enteritidis* and its LPS measured in Ussing chamber.	Same as above. Addition of histamines reversed this pattern.

2005; Yan *et al.* 2009; Hamilton *et al.* 2015), rats (Tan *et al.* 2015) and in *in vitro* studies, in chickens (Song *et al.* 2013, 2014). However, in chickens, FITC-d was measured by the Ussing chamber technique, which does not consider live-animal responses (circulatory, endocrine and inflammatory responses). Recently, FITC-d was used to measure increased IP in chicken blood samples (Tellez *et al.* 2014; Kuttappan *et al.* 2015; Vicuña *et al.* 2015). The FITC-d mechanism is similar to the paracellular pathway of the L sugar, as discussed above and shown in Fig. 2. However, there is some evidence of FITC-d being used in chickens in preference to the L-sugar method (Vicuña *et al.* 2015). Due to the different molecular sizes of L and FITC-d, future studies combining these methods may give further insight into TJ porosity.

Ussing chamber method

This is an *in vitro* technique where representative intestinal samples from different treatments are mounted on specially designed apparatus known as an Ussing chamber (Jeurissen *et al.* 2002). This method has been validated in chickens (Awad *et al.* 2008; Ruhnke et al. 2013). However, this method is an *in vitro* technique and does not consider live-animal responses, as mentioned above (Vicuña *et al.* 2015). Two measurements can be made with the Ussing chamber, namely, TEER and nutrient passage across intestinal segments. TEER measures ion permeability by electrical conductance and reduces when IP increases. This method has been used in pigs (Albin *et al.* 2007), mice (Guo *et al.* 2013), rats (Garnaut *et al.* 2002) and chickens (Song *et al.* 2013, 2014; Ruhnke *et al.* 2013; Awad *et al.* 2015) to measure IP. Nutrient passage across the epithelium in Ussing chambers has been used to show increased IP for FITC-d in chickens (Song *et al.* 2013, 2014), mannitol (Collier *et al.* 2003), glucose and serine transport (Ghareeb *et al.* 2015).

Tight-junction protein expression

Gene expression of TJ proteins is another indicator of increased IP and has been used in chickens (Table 5). The expression of ZO-1 protein and β-catenin protein in pigs has been used as a measure of increased IP (Klunker *et al.* 2013). TJ protein expression of ZO was decreased in chickens following an LPS treatment (Wang *et al.* 2014; Li *et al.* 2015). Similarly, expression of occludens and ZO-1 proteins was reduced in chickens following heat stress (Song *et al.* 2014) and mycotoxin challenge (Osselaere *et al.* 2013), showing increased IP. Protein expression could be informative for assessing increased IP, but requires animal tissues. Sugar methods have an advantage as they can be conducted in live animals. Additionally, transcription of the proteins (through mRNA expression) may not always be translated into 100% protein synthesis (translation phase; Vogel and Marcotte 2012).

Intestinal fatty acid-binding proteins (iFABP)

Intestinal fatty acid-binding proteins (iFABP) are 15 kD proteins found only in the enterocytes of the small intestine (Ni *et al.* 2015) as shown in Fig. 2. These proteins regulate fatty acid transport across the epithelial layer (Piton *et al.* 2015) and are released into the blood circulation as a result of mucosal

Table 5. Summary of the different biomarkers used for assessing intestinal permeability (IP)

Test	Basic measurements	Advantage(s)	Disadvantage (s)
Lactulose (L), L-rhamnose (R) and mannitol (M) sugars	Measures transcellular (R and M) and paracellular permeability (L)	1. Measures IP throughout the intestine. 2. Can be used in same animals repeatedly. 3. Less invasive technique.	1. Has not been conducted in chickens, but has been conducted in dogs, rabbits, rats, mice, birds and humans only. 2. Oral gavage needs to be given with sugars to birds and requires blood collection.
Fluorescein isothiocyanate dextran (FITC-d)	Measures paracellular permeability as above (L) method	1. Has been used in chickens recently. 2. Measures IP throughout the intestine. 3. Can be used in same animals repeatedly. 4. Less invasive technique.	Oral gavage needs to be given with sugars to birds and require blood collection.
Ussing chamber	Trans-epithelial electrical resistance and nutrient movement	1. Many measurements can be taken from small sections of the intestines. 2. Can specifically measure IP of duodenum, jejunum and ileum separately.	1. *In vitro* technique as does not include the responses from live animal. 2. Measures IP of a specific section of the intestine at one point. 3. Need special apparatus. 4. Need to kill birds to get tissues
Tight-junction (TJ) protein expression	Measures the transcriptional process of TJ protein expressions	Has been conducted in poultry and pigs.	1. Cannot be performed in same animals repeatedly. 2. Not a direct measure of IP. 3. Messenger RNA transcription may not always lead to full protein translation.
Intestinal fatty acid binding protein (iFABP) and antitrypsin inhibitor (AAT)	Measures IP due to the damage in mucosa	1. Less invasive technique. 2. Can be measured in blood. 3. Can be used in same animals repeatedly.	Has not been conducted in chickens so no data to compare results.
D-lactate	Measures IP as lactate passes from intestine to blood	1. Less invasive technique. 2. Can be measured in blood. 3. Can be used in same animals repeatedly.	1. Only a few studies have been conducted in chickens. 2. Has not been used in disease- or stress-challenged chickens.
Diamine oxidase (DAO)	Measures IP as DAO passes from intestinal mucosa to blood	1. Less invasive technique. 2. Can be measured in blood. 3. Can be used in same animals repeatedly. 4. Has been used in intestinally compromised chickens.	Few studies have been conducted in chickens.
Bacterial translocation	Measures IP as pathogens pass from intestinal mucosa to blood	Has been used in chickens.	Need to kill birds to collect tissues.
LPS in blood	Measures IP when endotoxins of pathogens traverse from the intestinal mucosa to blood	Less invasive technique as it can be measured in blood.	1. Has been used in only one study. 2. The model used in the study suggests that it requires further experiments to use this model in a variety of conditions.

damage following mesenteric ischemia or intestinal necrosis (He *et al.* 2014), intestinal ischemia (Shi *et al.* 2015) and sepsis (Piton *et al.* 2015). Mesenteric ischemia in pigs leads to reduced blood supply to the intestine, leading to increased IP measured by iFABP (He *et al.* 2014). Intestinal ischemia in humans, caused by either mesenteric or intestinal obstruction, leads to increased IP measured by iFABP (Shi *et al.* 2015). Sepsis is a gastrointestinal disease of humans that damages enterocytes and increases IP (Piton *et al.* 2015). Sepsis in rats also increases IP which was measured by increased iFABP in serum by Shen *et al.* (2015). Serum iFABP was also increased in rats when IP was increased by ischemia, which was confirmed by reduced mRNA expression of TJ (Rosero *et al.* 2014).

Ni *et al.* (2015) also found that a treatment with extracorporeal membrane oxygen in infants during acute respiratory disease increased serum iFABP. Extracorporeal membrane oxygen has been known to increase IP in piglets (Kurundkar *et al.* 2010), suggesting that increased serum iFABP was due to increased IP. In a similar study in rats, omega-3 fatty acids reduced IP and serum iFABP. Reduction in IP was also confirmed by reduced endotoxins and upregulation of TJ protein expression (Li *et al.* 2014). These studies showed that iFABP is a useful indicator of increased IP in humans, rats and pigs. There are different types of iFABP including FABP-1, FABP-2 and FABP-10 (Niewold *et al.* 2004; Chen *et al.* 2015*a*; Niewold 2015; Shi *et al.* 2015). The chicken iFABP gene is 66% similar to the pig gene (Niewold 2015), suggesting that increased iFABP in serum would indirectly reflect increased IP in chickens. In a recent study, downregulation of iFABP-2 gene was associated with an increase in IP in chickens (Chen *et al.* 2015*a*). IFABP detection in blood is an easy alternative; however, this has yet to be studied in chickens.

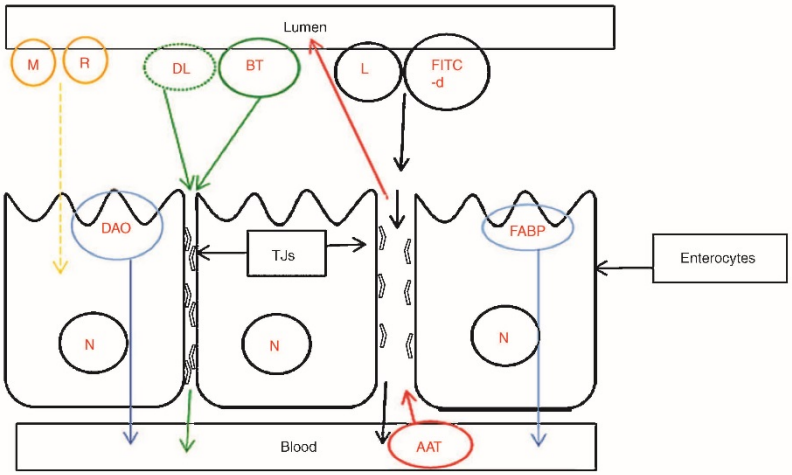

Fig. 2. Presentation of different biomarkers to assess increased intestinal permeability. M, mannitol; R, rhamnose (dotted arrow through enterocyte shows trans-cellular pathway from lumen to blood); L, lactulose; FITC-d, fluorescence isothiocyanate dextran (solid arrow through damaged tight junctions shows paracellular pathway from lumen to blood); DL, D-lactate; BT, bacterial translocation or endotoxins (with solid arrows); FABP, intestinal fatty acid-binding protein found in the enterocytes that can enter blood due to increased intestinal permeability (IP); DAO, diamine oxidase found in enterocytes that can enter blood due to increased IP; AAT = α antitrypsin inhibitor found in blood that can enter the lumen due to increased IP; and TJs, tight junctions.

α-1 antitrypsin inhibitor protein (AAT)

Alpha-1 antitrypsin inhibitor protein (AAT) is a glycoprotein that is synthesised in the liver to protect tissues during inflammation (Parambeth *et al.*2015). It is found in serum and in very low quantities in the lumen or faeces of healthy animals (Burke *et al.* 2012). However, when the intestinal barrier function is compromised, AAT can cross the mucosal barrier (Mohr *et al.* 2003; Murphy *et al.* 2003). Since AAT is not metabolised by the microbes in the intestine, it can be measured in the faeces (Parambeth *et al.* 2015). AAT is an established biomarker of leaky gut in humans (Kosek *et al.* 2013; Colman and Rubin 2014). Additionally, AAT found in faeces shows protein loss (due to trans-mucosal leakage from blood to the lumen) in cats (Burke *et al.* 2012) and dogs (Parambeth *et al.* 2015) and an increased IP in dogs (Suchodolski *et al.* 2012). Although AAT in chickens is similar to that in humans, no published literature can be found on this test in relation to IP in chickens (Niewold 2015). Only one article shows increased AAT values in chicken serum rather than excreta, following a coccidiosis challenge (Golab *et al.* 2007). This suggests that during inflammation, AAT is produced in chickens; however, as explained above, its measurement in excreta is yet to be determined.

D-lactate in serum

D-lactate is produced by intestinal bacteria as a product of fermentation (Shi *et al.* 2015) and can traverse the intestinal barrier due to increased IP in humans (Ni *et al.* 2015; Xun *et al.* 2015). Since D-lactate cannot be metabolised in the liver, its increased level in serum due to intestinal ischemia (Shi *et al.* 2015) and colitis (Sun *et al.* 2015) has been used as a biomarker of increased IP. In a piglet model, increased IP was measured by increased bacterial translocation and elevated concentrations of D-lactate in serum (Kurundkar *et al.* 2010). Recently, a reduced D-lactate concentration was linked with decreased IP in pigs (Xun *et al.* 2015). In rats, increased IP was measured by downregulation of mRNA expression of ZO1 and was comparable with increased concentration of serum D-lactate (Rosero *et al.* 2014). In another study in rats, reduced D-lactate was linked with improved IP (Tan *et al.* 2015). D-lactate has been observed in laying hens as a fermentation by-product in the ileum (Meyer *et al.* 2013). Similarly, serum D-lactate has been used as a biomarker of increased IP in laying hens (Lei *et al.* 2013). However, chickens were not challenged in these studies. In the above studies, D-lactate values were decreased in chickens fed feed additives (probiotic and zeolites), compared with control birds. Only one study showed that D-lactate concentrations increased due to LPS challenge in broilers (Wu *et al.* 2013). The concentrations of D-lactate in serum during a disease or a stress challenge would, therefore, be interesting to investigate in relation to IP in chickens.

Diamine oxidase activity in serum (DAO)

Diamine oxidase (DAO) is an endo-cellular enzyme of 250 kD that catalyses the deamination of histamines such as diamines through oxidation (Wu *et al.* 2013). Histamines are produced during intestinal ischemia and stress (Zhang *et al.* 2015). DAO

is localised in the intestinal mucosal cells in mammals and enters the circulation through the damaged intestinal barrier (He *et al.* 2014; Sun *et al.* 2015; Xun *et al.* 2015). Increased serum DAO and iFABP have been linked with increased IP in heat-stroke patients, and decreased linearly after treatment (Zhang *et al.* 2015). Increased DAO concentrations for increased IP have been observed in LPS-challenged rats (Ruan *et al.* 2014), colitis in rats (Sun *et al.* 2015) and pigs (Liu *et al.* 2012). Furthermore, reduced DAO concentrations have been linked with decreased IP in pigs (Xun *et al.* 2015). In chickens, feeding probiotics (Lei *et al.* 2013) and zeolite (Wu *et al.* 2013) decreased serum DAO concentrations, representing decreased IP. Additionally, LPS-challenged broiler birds exhibited increased serum DAO concentrations, indicating increased IP (Wu *et al.* 2013; Li *et al.* 2015). Table 5 shows that this biomarker has recently been used in chickens. However, further studies are required to confirm its practicability.

Bacterial translocation and serum endotoxin concentrations

Bacteria passing through the TJ of the intestine can reach the liver and induce inflammation (Ilan 2012), which can alter energy partitioning (Shen *et al.* 2015). In rats, increased bacterial translocation was observed due to ischemia. Ischemia also increased IP in these rats, measured by increased D-lactate and iFABP concentrations (Rosero *et al.* 2014). An increase in bacterial translocation has also been observed in heat-stroke patients where IP was increased initially, but decreased following cooling and antibiotic treatment (Zhang *et al.* 2015). Bacterial translocation has been measured in the liver as a biomarker of increased IP in turkeys (Tellez *et al.* 2015) and broilers (Tellez *et al.* 2014). Table 5 illustrates that this method requires tissues from animals, compared with other, non-invasive, techniques such as the sugar method.

Similar to bacterial translocation, the endotoxins of bacteria (LPS) can penetrate the intestinal barrier (Chen *et al.* 2015*a*). However, endotoxin measurement might be less useful where LPS is being used as a model to increase intestinal permeability.

Summary of biomarkers

As shown in Table 5, different biomarkers can be used to measure increased IP in chickens. Although biomarkers such as TJ protein expression and bacterial translocation have been used in chickens, these require animal tissues. AAT is potentially a non-invasive biomarker, as it can be detected in faeces, but it has not yet been explored in chickens. The sugar method and iFABP are less invasive, but they have yet to be explored in chickens. Other biomarkers, which include FITC-d, DAO and D-lactate, require blood specimens (semi-invasive) and have recently been used in chickens. However, further studies are required to confirm their validity in future studies.

Conclusions

In vivo studies of IP in chickens have been limited. Recently, models such as fasting, dextran sodium sulfate and LPS have been used to increase IP in chickens; however, these will require more rigorous research before they can be readily applied. There is potential for LPS to act as a model to increase IP in chickens, on the basis of the recent research discussed in section *Lipopolysaccharide (LPS)*. There are currently a few biomarkers used to assess increased IP in chickens. The use of FITC-d sugars is showing promise, although further research is required to validate this biomarker. The sugar-recovery method also has the potential to be used in chickens as a biomarker of IP. Since FITC-d and lactulose sugars are of different sizes, their translocation can give insight into TJ porosity. The development of different models of compromised intestinal barrier function, together with the validation of specific and sensitive biomarkers, will aid our understanding of responses to stress and diseases, and will improve our ability to maintain chicken health through targeted interventions.

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Chapter 3: Intestinal permeability induced by lipopolysaccharide and measured by lactulose, rhamnose and mannitol sugars in chickens

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Overall percentage (%)	75		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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ii. permission is granted for the candidate in include the publication in the thesis; and

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Intestinal permeability induced by lipopolysaccharide and measured by lactulose, rhamnose and mannitol sugars in chickens

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Increased intestinal permeability (IP) can lead to compromised health. Limited in vivo *IP research has been conducted in chickens. The objectives of the current study were to develop a model of increased IP utilizing lipopolysaccharide (LPS* Escherichia coli *O55: B5) and to evaluate IP changes using the lactulose, mannitol and rhamnose (LMR) sugar permeability test. In addition, fluorescein isothiocyanate dextran (FITC-d), D-lactate, zonula occludens (ZO-1) and diamine oxidase (DAO) permeability tests were employed. Male Ross chickens were reared until day 14 on the floor in an animal care facility and then transferred to individual cages in three separate experiments. In each of experiments 1 and 2, 36 chicks were randomly allocated to receive either saline (control) or LPS (n = 18/group). Lactulose, mannitol and rhamnose sugar concentration in blood was measured at 0, 30, 60, 90, 120 and 180 min in experiment 1, at 60, 90 and 120 min in experiment 2 and at 90 min in experiment 3 (n = 16/group). Lipopolysaccharide was injected intraperitoneally at doses of 0.5, 1 and 1 mg/kg BW in experiments 1, 2 and 3, respectively, on days 16, 18 and 20, whereas control received sterile saline. On day 21, only birds in experiments 1 and 2 were fasted for 19.5 h. Chicks were orally gavaged with the LMR sugars (0.25 gL, 0.05 gM, 0.05 gR/bird) followed by blood collection (from the brachial vein) as per time point for each experiment. Only in experiment 3, were birds given an additional oral gavage of FITC-d (2.2 mg/ml per bird) 60 min after the first gavage. Plasma D-lactate, ZO-1 and DAO concentrations were also determined by ELISA in experiment 3 (n = 10). Administration of LPS did not affect IP as measured by the LMR sugar test compared with control. This was also confirmed by FITC-d and DAO levels in experiment 3 ($P > 0.05$). The plasma levels of D-lactate were decreased ($P < 0.05$). Plasma levels of ZO-1 were increased in the third experiment only and did not change in the first two experiments. Lipopolysaccharide at doses of 0.5 and 1 mg/kg did not increase IP in this model system. In conclusion, the LMR sugar can be detected in blood 90 min after the oral gavage. Further studies are needed for the applicability of LMR sugars tests.*

Keywords: lactulose, rhamnose, mannitol, lipopolysaccharide, FITC-d

Implications

Increased intestinal permeability (IP) can lead to health disorders and reduced feed efficiency in chickens. We aimed to develop a model of increased IP by administering gram-negative bacterial toxin lipopolysaccharide (LPS) to chickens. This model could then be applied in an attempt to develop a new lactulose, mannitol and rhamnose (LMR) sugar permeability method to determine IP. An established model of increased IP and novel biomarkers to monitor IP changes could be applied to the study of nutritional interventions aimed at improving the health and welfare of chickens. However, further research is required to determine the optimal LPS dose.

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Introduction

Increased IP results when enterocytes linked by tight junctions (TJs) fail to prevent microbes or toxins entering the systemic circulation (Li *et al.*, 2015). Human studies of increased IP have been associated with enteric infections, inflammatory bowel disease, obesity, type-1 diabetes, liver diseases, irritable bowel syndrome and celiac disease (Skouroliakou *et al.*, 2016). Limited *in vivo* research has shown that increased IP can lead to increased bacterial

translocation (Chen *et al.*, 2015), coccidiosis (Chapman, 2014) and lameness (Wideman *et al.*, 2012) in chickens. These diseases can impact health and welfare leading to reduced efficiency and economic loss (Zuidhof *et al.*, 2014).

Increased IP models are well established in humans (Ni *et al.*, 2015; Piton *et al.*, 2015), pigs (Zhu *et al.*, 2015), rats and mice (Williams *et al.*, 2013; Ruan *et al.*, 2014). Dextran sodium sulphate (DSS) ingestion, fasting (Kuttappan *et al.*, 2015; Vicuna *et al.*, 2015b), dexamethasone (Vicuna *et al.*, 2015a) and wire floor stress (Wideman *et al.*, 2012) have recently been employed as models of increased IP in chickens. These studies have been conducted in one strain of chickens (Cobb). However, it has been determined that the IP of different strains is affected differently by DSS ingestion (Perše and Cerar, 2012). Lipopolysaccharide, found in the outer coat of gram-negative bacteria (Tran, 2009), has been well established as a model to increase IP in mice (Williams *et al.*, 2013), rats (Ruan *et al.*, 2014) and pigs (Zhu *et al.*, 2015). Although LPS has been used to induce systemic inflammation in chickens (Hu *et al.*, 2011; Tan *et al.*, 2014), limited research has been conducted regarding its effect on IP as reviewed by Gilani *et al.* (2016). Lipopolysaccharide, at doses of 0.25 to 1 mg/kg BW, has been used to increase IP in chickens (Wu *et al.*, 2013; Li *et al.*, 2015).

In addition, there is limited literature of *in vivo* biomarkers that could potentially be utilized to evaluate IP changes in chickens (Gilani *et al.*, 2016). Lactulose, rhamnose and mannitol sugars have been validated in rats (Ruan *et al.*, 2014), pigs (Wijtten *et al.*, 2011) and humans (Cox *et al.*, 1999; van Wijck *et al.*, 2013; Tran *et al.*, 2015), and could be applied to chickens. Lactulose, a high molecular weight (342 Da) sugar, traverses the epithelium paracellular, through the TJs of enterocytes in small quantities. Rhamnose and mannitol are lower molecular weight (182 Da) sugars which are transcellular absorbed directly into the blood. Increased lactulose to rhamnose (van Wijck *et al.*, 2013; Tran *et al.*, 2015) or L/M ratio (Araujo *et al.*, 2015; Shaikh *et al.*, 2015) due to increased permeability of lactulose and reduced absorbance of rhamnose or mannitol (due to damaged surface area for absorption) represents a biomarker of increased IP.

Recently, diamine oxidase (DAO), D-lactate (Wu *et al.*, 2013; Li *et al.*, 2015) and fluorescein isothiocyanate dextran (FITC-d) (Vicuna *et al.*, 2015b) concentrations in blood have been utilized as *in vivo* biomarkers of increased IP in chickens. In addition, increased concentrations of zonula occludens (ZO-1) have been observed in celiac disease, diabetic and insulin-resistant human patients (Klaus *et al.*, 2013). The aims of the current study were to evaluate whether the LMR sugar test could be used to evaluate changes in permeability following LPS administration at doses of 0.5 mg/kg BW (with fasting) and 1 mg/kg BW (with and without fasting) in chickens. The second aim was to compare the LMR sugar test with blood concentrations of FITC-d, DAO, D-lactate and ZO-1 as potential biomarkers of increased IP.

Material and methods

Animal management

Day-old male Ross 308 broiler chicks were obtained from a local hatchery (Baiada, Gawler, Australia) and raised on indoor floor pens on wood shavings for 14 days. Birds were fed a commercially available broiler feed (Ridley Agri Products Pty., Murray Bridge, SA, Australia). All birds were provided with *ad libitum* feed and water with 16 h day and 8 h night cycle. The birds were randomly allocated to two groups, control and LPS treatment. On day 14, birds were allocated in pairs to metabolic cages for 2 days. After 2 days acclimatization, birds were weighed and administered either LPS (*Escherichia coli* 055:B5; Sigma-Aldrich, Sydney, Australia) or sterile saline *intraperitoneally* at 16, 18 and 20 days of age.

Experimental protocol

All protocols were approved by the Animal Ethics Committees of the University of Adelaide and the Primary Industry Regions South Australia. All animal studies were performed in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Oral solutions and gavages

Birds were orally gavaged via a feeding tube (round tip at the end) fitted to a disposable syringe. The tube was carefully inserted ~6 to 8 cm into the oesophagus. Lactulose, mannitol and rhamnose sugar solution was prepared with 25 g lactulose, 5 g mannitol and 5 g rhamnose (Sigma-Aldrich) dissolved in 100 ml milli-Q water (MilliPore, Molsheim, France; 18.2 MΩ cm at 25°C). Fluorescein isothiocyanate dextran (4000 Da molecular weight; Sigma-Aldrich) was dissolved in milli-Q water (2.2 mg FITC-d in 1 ml).

Experiment 1

In the first experiment, 36 birds were distributed into control and LPS groups and were intraperitoneally administered either LPS or saline at 0.5 mg/kg BW on days 16, 18 and 20. Birds in each group were distributed further into sub-groups according to their blood collection time points of 0, 30, 60, 90, 120, 180 min ($n = 3$ per time point). Following their final injection, birds in both groups were fasted for 19.5 h (overnight). The next day, (day 21) all groups except time point 0 were orally gavaged with LMR sugar solution (2 ml/bird) and blood collected from the brachial vein.

Experiment 2

In the second experiment ($n = 36$), similar procedures to the first experiment were followed except for the LPS dose and time points. The dose of LPS in this experiment was increased to 1 mg/kg BW and birds in the control and LPS groups were distributed only into three time points of 60, 90 and 120 min ($n = 6$ per time point). Oral gavage and blood collection procedures were as for the first experiment.

Experiment 3

In the final experiment, 32 birds were randomly allocated into control and LPS groups and were intraperitoneally administered 1 mg/kg BW LPS or sterile saline. Only one time point (90 min) was used ($n = 16$). Contrary to the first two experiments, birds were not fasted. In addition, birds were gavaged twice, first with FITC-d at time 0 min (2.2 mg/ml per bird) and second with LMR sugar at 60 min (2 ml/bird). Blood was collected from the brachial vein at 90 min after LMR gavage, and then allocated to serum and plasma vacutainers (BD, Sydney, NSW, Australia).

Processing blood samples

After collection, blood samples for plasma and serum were placed on ice and at room temperature, respectively, and centrifuged at 3000 r.p.m. for 10 min. The supernatant was collected and plasma and serum were stored in 0.5 aliquots at −20°C until further analysis.

High-performance ionic chromatography analysis

Lactulose, mannitol and rhamnose standards were prepared using fasted chicken plasma (sourced from a local slaughter house), and spiked to the following concentrations 125, 62.5 and 31.25 μM and stored at −20°C until further analysis. After thawing plasma and samples, 15% tri-chloroacetic acid (TCA; Sigma-Aldrich) was added, centrifuged at 13 000 r.p.m. for 10 min and the supernatant mixed with an equal volume of mixed-bed ion exchange matrix amberlite MB-1 resin (Sigma-Aldrich) for 30 min. The supernatant was transferred to Thomson single step™ 0.2 μm nylon filter vials (Adelab Pty Ltd, Adelaide, SA, Australia) and analysed using the Dionex ICS-4000 (Thermo Fisher Pty Ltd, Scoresby, Victoria, Australia) high-performance ionic chromatography (HPIC) system as described by Tran *et al.* (2015).

Fluorescein isothiocyanate dextran, diamine oxidase, D-lactate and zonula occludens tight junction proteins in blood

Fluorescein isothiocyanate dextran analysis was conducted as previously described (Vicuna *et al.*, 2015b). In brief, standards (0, 0.0001, 0.001, 0.01, 0.1, 1.0 and 10 μg/ml) were prepared using the same FITC-d sugars as for oral gavage. Standards and serum samples were run in triplicate and concentration of FITC-d in serum was calculated by a standard curve and a Synergy MX plate reader (BioTek Instruments, Bedfordshire, UK) at excitation and emission wavelengths of 485 and 530 nm, respectively. ELISA kits for DAO, D-lactate and TJ proteins ZO-1 were obtained from MyBioSource, Inc. (San Diego, California, USA). All standards and plasma samples were measured in duplicate in a microplate reader at 450 nm wavelength (Bio-Rad Laboratories Inc., California, USA) following the manufacturer's instructions.

Statistical analysis

All statistical analyses were conducted using SPSS 22 (IBM SPSS; IBM Corp., Armonk, New York, USA). BW, concentrations of individual sugars (LMR) (μmol/l) and ratios (L/R and L/M), FITC-d, DAO, D-lactate and TJs were compared using one-way ANOVA. Interaction between LPS dose and time points were measured by two-way ANOVA. Data from all experiments were normally distributed and significance (P) was measured at 0.05.

Results

BW

The BW increment of the LPS-treated group were significantly lower than control during days 16 to 18 for all three experiments and during 18 to 20 days in the first experiment only (Table 1). In addition, at day 16, BW of control and LPS-treated birds were not different significant in all three experiments (data not shown).

Lactulose, rhamnose and mannitol concentrations and their ratios

Lactulose, rhamnose and mannitol sugar concentrations in blood (μmol/l) for control and LPS treatment groups were not significantly different (Table 2). However, rhamnose concentration was significantly higher at 180 min for the LPS-treated group in the first experiment compared with the control group. Similarly, rhamnose and mannitol values were significantly lower in the third experiment in LPS-treated group compared with the control group.

The ratios of L/R and L/M were not statistically different between control and LPS treatment in experiments 1 and 2 (Table 3). However, the L/R ratio in the third experiment was significantly higher in the LPS-treated group compared with control due to significantly lower concentration of rhamnose in the LPS-treated group. Although mannitol concentration was also significantly lower in the third experiment, it did not change the L/M ratio.

Lactulose, rhamnose and mannitol levels peaked at 90 to 120 min in the blood post oral gavage in the first experiment (0, 30, 60, 90, 120 and 180 min) as shown in Figure 1. This

Table 1 *Average BW increment ± SEM of control and lipopolysaccharide (LPS)-treated chickens during 16 to 18 and 18 to 20 days of age*

Experiments	1 ($n = 18$)		2 ($n = 18$)		3 ($n = 16$)	
LPS dose (mg/kg)	0.5		1		1	
Fasting (h)	19.5		19.5		0 (*ad libitum* feed)	
Age (days)	16 to 18	18 to 20	16 to 18	18 to 20	16 to 18	18 to 20
BW (g)						
Control	177 ± 3.9^{a}	175 ± 4.9^{a}	155 ± 4.5^{a}	168 ± 5.8^{a}	159 ± 3.8^{a}	180 ± 5.5^{a}
LPS	143 ± 3.5^{b}	157 ± 6.7^{b}	115 ± 6.7^{b}	153 ± 7.1^{a}	133 ± 5.3^{b}	177 ± 4.2^{a}

[a,b]Different superscripts show the significance level $P < 0.05$.

Table 2 *Chicken plasma values of lactulose, rhamnose and mannitol (μmol/l) (mean ± SEM) in three experiments*

			Lactulose		Rhamnose		Mannitol	
Experiment number	Time (min)	*n*	Control	LPS	Control	LPS	Control	LPS
1	0	3	0	0	0	0	0	0
	30	3	1.8 ± 0.5	2.7 ± 0.5	22.5 ± 4.8	29.8 ± 4.8	20.2 ± 2.4	23.1 ± 2.4
	60	3	2.8 ± 0.5	2.2 ± 0.5	32.0 ± 4.8	33.1 ± 4.8	25.7 ± 2.4	25.7 ± 2.4
	90	3	2.5 ± 0.5	2.5 ± 0.5	31.9 ± 4.8	32.9 ± 4.8	28.4 ± 2.4	22.6 ± 2.4
	120	3	2.7 ± 0.5	3.1 ± 0.5	24.4 ± 4.8	27.8 ± 4.8	22.1 ± 2.4	22.0 ± 2.4
	180	3	1.4 ± 0.5	2.7 ± 0.5	13.2 ± 4.8[a]	24.9 ± 4.8[b]	14.2 ± 2.4	20.7 ± 2.4
2	60	6	4.1 ± 0.7	5.7 ± 0.7	26.3 ± 2.9	25.4 ± 2.9	21.1 ± 2.1	15.3 ± 2.1
	90	6	4.0 ± 0.7	4.1 ± 0.7	19.5 ± 2.9	23.4 ± 2.9	18.1 ± 2.1	18.2 ± 2.1
	120	6	4.5 ± 0.7	3.5 ± 0.7	16.7 ± 2.9	15.0 ± 2.9	18.3 ± 2.1	17.6 ± 2.1
3	90	16	3.6 ± 1.1[a]	3.2 ± 1.9[a]	15.3 ± 0.77[a]	10.7 ± 0.64[b]	21.2 ± 1.0[a]	17.8 ± 1.0[b]

LPS, lipopolysaccharide.
[a,b]All values are $P > 0.05$ except where stated as different superscripts.

Table 3 *Chicken plasma lactulose to rhamnose (L/R) and mannitol (L/M) ratios (mean ± SEM)*

			L/R ratio		L/M ratio	
Experiment number	Time (min)	*n*	Control	LPS	Control	LPS
1	0	3	0	0	0	0
	30	3	0.07 ± 2.06	0.10 ± 2.06	0.08 ± 2.14	0.12 ± 2.14
	60	3	0.10 ± 2.06	0.07 ± 2.06	0.12 ± 2.14	0.09 ± 2.14
	90	3	0.08 ± 2.06	0.08 ± 2.06	0.09 ± 2.14	0.11 ± 2.14
	120	3	0.11 ± 2.06	0.11 ± 2.06	0.12 ± 2.14	0.14 ± 2.14
	180	3	0.11 ± 2.06	0.11 ± 2.06	0.10 ± 2.14	0.13 ± 2.14
2	60	6	0.15 ± 5.1	0.26 ± 5.1	0.19 ± 4.8	0.38 ± 4.8
	90	6	0.23 ± 5.1	0.19 ± 5.1	0.24 ± 4.8	0.24 ± 4.8
	120	6	0.31 ± 5.1	0.25 ± 5.1	0.26 ± 4.8	0.22 ± 4.8
3	90	16	0.24 ± 0.01[a]	0.32 ± 0.03[b]	0.18 ± 0.01	0.20 ± 0.02

LPS, lipopolysaccharide.
[a,b]All values are $P > 0.05$ except where stated with different superscripts.

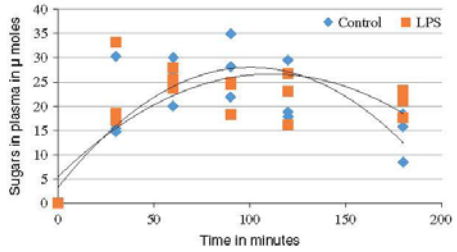

Figure 1 Lactulose, rhamnose and mannitol sugars in chicken plasma at 0, 30, 60, 90, 120 and 180 min ($n = 3$ per time point) post oral gavage. LPS, lipopolysaccharide.

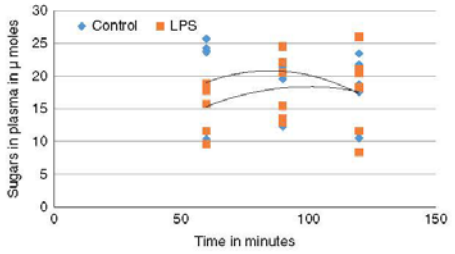

Figure 2 Lactulose, rhamnose and mannitol sugars in chicken plasma at 60, 90 and 120 min ($n = 6$ per time point) post oral gavage. LPS, lipopolysaccharide.

was repeated in the second experiment, with only three time points of 60, 90 and 120 min, as shown in Figure 2, confirming the trend that the optimal time point for collecting sugars was 90 min post oral gavage.

Zonula occludens concentration for LPS-treated chickens was not significantly different than control in the first two experiments. However, ZO-1 concentration in the third experiment was significantly higher for LPS-treated chickens (Table 4). Diamine oxidase, D-lactate and FITC-d concentrations were measured in the third experiment only. D-lactate values decreased significantly due to the LPS treatment. Fluorescein isothiocyanate dextran and DAO were not significantly different for LPS treatment compared with control (Table 4).

Table 4 *Zonula occludens, diamine oxidase (DAO), D-lactate and fluorescein isothiocyanate dextran (FITC-d) (mean ± SEM) of control and lipopolysaccharide (LPS) treated chickens*

Experiment number	Test	*n*	Units	Control	LPS
1	ZO-1	10	pg/ml	121.2 ± 78.8	86.66 ± 20.2
2	ZO-1	15	pg/ml	137.7 ± 25.29	129.5 ± 17.7
3	ZO-1	10	pg/ml	643.6 ± 43.0[a]	900.1 ± 33.2[b]
3	FITC-d	15	µg/ml	1.91 ± 0.04	1.81 ± 0.03
3	D-lactate	10	µg/ml	4.04 ± 0.22[a]	2.46 ± 0.32[b]
3	DAO	10	ng/ml	12.71 ± 0.72	12.62 ± 0.91

[a,b]All values are $P > 0.05$ except where stated as different superscripts.

Discussion

This is the first study to utilize the lactulose, rhamnose and mannitol sugar test to evaluate IP changes following LPS challenge in chickens. The concentrations of LMR (and their ratios) in the first two experiments did not indicate any IP changes following LPS treatment. The interaction between the LPS dose and time points for individual sugars was not statistically significant. In addition, birds in these experiments were fasted for 19.5 h and recently it has been shown that 24 h fasting increased IP in chickens (Kuttappan *et al.*, 2015; Vicuna *et al.*, 2015b). To exclude the possible interference of fasting with LPS, a third experiment at 1 mg/kg BW without fasting was conducted. The L/R ratio for the LPS treatment was significantly increased compared with control, unlike the L/M ratio. Araujo *et al.* (2015) and Shaikh *et al.* (2015) argued that L/M and L/R ratios should be considered along with the plasma concentrations of the individual sugars to better understand the sugar permeability test. The increased L/R ratio in the current study was due to the decreased rhamnose concentration, rather than increased lactulose permeation. In addition, rhamnose and mannitol are of similar molecular size, suggesting that both sugars and their ratios should behave similarly with respect to permeation (Wang *et al.*, 2015). However, in no other studies have both sugars been studied together. In summary, the unaffected lactulose concentrations in the current study suggested that fasting did not obscure LPS effects; and LPS at doses of 0.5 and 1 mg/kg BW did not provide a reliable method to increase IP in chickens. Before gavage with sugars, blood plasma did not contain lactulose, rhamnose or mannitol (Figure 1), indicating no endogenous sugars in the blood. The plasma concentrations showed peaks for mannitol and rhamnose at 90 min and a peak concentration for lactulose at 120 min. In other time-course experiments these sugars peaked at 120 min in dogs (Sørensen *et al.*, 1997) and in humans (Cox *et al.*, 1999), comparable with the current lactulose results, but not for rhamnose and mannitol.

In the current study, BW decreased after the first LPS injection in all three trials consistent with previous poultry studies (Hu *et al.*, 2011; Tan *et al.*, 2014). Although feed intake was not measured in these trials, others have shown that LPS induced a systemic inflammation and reduced feed intake (Wu *et al.*, 2013). This may have been the reason for the reduced BW observed in the current study. However, BW did not reduce after the second LPS injection, consistent with other studies (Shini *et al.*, 2008; Wu *et al.*, 2013). It is possible that chickens in the LPS treatment group may have overcome their initial shock resulting in similar growth between both groups. This may also explain why the sugar permeability test did not show statistical difference between control and LPS treatment.

Diamine oxidase is an endo-cellular enzyme of 250 kDa (Sun *et al.*, 2015) and has been utilized in rats for increased IP (Ruan *et al.*, 2014), pigs (Xun *et al.*, 2015) and chickens (Lei *et al.*, 2013; Wu *et al.*, 2013; Li *et al.*, 2015). Diamine oxidase concentrations in the third experiment for control and LPS treatment were inconsistent with previous studies. This may have been due to mild intestinal inflammation induced by LPS as previously reported (Wang *et al.*, 2012). In addition, different strains of chickens used (Arbor Acres *v.* Ross 308 in the current study) may have also had an effect. D-lactate is produced by bacterial fermentation and have been employed in humans (Ni *et al.*, 2015; Shi *et al.*, 2015) and in chickens (Wu *et al.*, 2013). D-lactate concentration in the current study was not consistent with the previous study and could have been due to different strains of chickens, or different ELISA kits (porcine origin compared with chicken-specific kit in the current study).

Zonula occludens plays an important role in IP (Fink, 2003). Zonula occludens values, in the first two experiments were not significantly different compared with control, whereas increased significantly in the third experiment. These variations and limited published data may need further investigations. Fluorescein isothiocyanate dextran has a high molecular weight of 40 kDa and increased serum concentrations indicate increased IP in chickens (Kuttappan *et al.*, 2015; Vicuna *et al.*, 2015a and 2015b). However, DSS ingestion and fasting were utilized as models of increased IP, compared with the LPS model in the current studies. Fluorescein isothiocyanate dextran concentrations in the third experiment revealed that LPS at 1 mg/kg BW did not increase IP.

Conclusions

The lactulose, rhamnose and mannitol sugar concentrations in plasma did not indicate increased IP due to the LPS treatment. As LPS and the sugar test have not been previously utilized in poultry, DAO, D-lactate and FITC-d biomarkers were used to confirm that LPS at a dose of 1 mg/kg BW did not increase IP in the current study. In addition, it was noted that fasting of 19.5 h did not change the outcome of LPS administration on IP. Further studies are required to validate the lactulose, rhamnose and mannitol sugar permeability tests and to determine effective dose and LPS administration procedure to increase IP.

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Chapter 4: New biomarkers for increased intestinal permeability induced by dextran sodium sulphate and fasting in chickens

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Contribution to the Paper	Performed literature search, planned and conducted the experiments, analysed the blood samples and data, wrote the manuscript and acted as corresponding author		
Overall percentage (%)	75		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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ORIGINAL ARTICLE

New biomarkers for increased intestinal permeability induced by dextran sodium sulphate and fasting in chickens

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Summary

Increased intestinal permeability (IP) can lead to compromised health in chickens. As there is limited literature on *in vivo* biomarkers to assess increased IP in chickens, the objective of this study was to identify a reliable biomarker of IP using DSS ingestion and fasting models. Male Ross chickens (n = 48) were reared until day 14 on the floor pen in an animal care facility, randomized into the following groups: control, DSS and fasting (each with n = 16), and then placed in metabolism cages. DSS was administered in drinking water at 0.75% from days 16 to 21, while controls and fasted groups received water. All birds had free access to feed and water except the birds in the fasting group that were denied feed for 19.5 h on day 20. On day 21, all chickens were given two separate oral gavages comprising fluorescein isothiocyanate dextran (FITC-d, 2.2 mg in 1 ml/bird) at time zero and lactulose, mannitol and rhamnose (LMR) sugars (0.25 g L, 0.05 g M and 0.05 g R in 2 ml/bird) at 60 min. Whole blood was collected from the brachial vein in a syringe 90 min post-LMR sugar gavage. Serum FITC-d and plasma LMR sugar concentrations were measured by spectrophotometry and high-performance ion chromatography respectively. Plasma concentrations of intestinal fatty acid binding protein, diamine oxidase, tight junction protein (TJP), D-lactate and faecal α-antitrypsin inhibitor concentration were also analysed by ELISA. FITC-d increased significantly ($p < 0.05$) after fasting compared with control. L/M and L/R ratios for fasting and L/M ratio for DSS increased compared with control chickens ($p < 0.05$). TJP in plasma was significantly increased due to fasting but not DSS treatment, compared with controls. Other tests did not indicate changes in IP ($p > 0.05$). We concluded that FITC-d and LMR sugar tests can be used in chickens to assess changes in IP.

Keywords leaky gut, fluorescein isothiocyanate dextran, lactulose/rhamnose ratio, lactulose/mannitol ratio, D-lactate, diamine oxidase

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Introduction

Enterocytes linked by tight junctions (TJs) regulate absorption of nutrients and bacterial translocation and when this barrier is compromised it is known as increased intestinal permeability (IP) (Wang et al., 2014; Li et al., 2015). Increased IP has been well studied and reviewed in rats (Ruan et al., 2014; Hamilton et al., 2015), pigs (Ghareeb et al., 2014; Boyer et al., 2015) and humans (Piton et al., 2015; Shi et al., 2015; Tran et al., 2015; Wang et al., 2015; Zhang et al., 2015). Increased IP has been associated with enteric infections, inflammatory bowel disease, obesity, type 1 diabetes, liver diseases, irritable bowel syndrome and coeliac disease in humans as summarized by Skouroliakou et al. (2016). Until recently, only few *in vivo* studies have investigated IP in chickens. These studies included the effects of rye-based diets due to their higher concentration of non-starch polysaccharides (Tellez et al., 2014), high dose of coccidiosis vaccine (Chen et al., 2015), dextran sodium sulphate (DSS) (Kuttappan et al., 2015) and fasting (Vicuna et al., 2015b) on changes in IP in chickens. Increased IP in chicken may potentially lead to bacterial translocation (Vicuna et al., 2015a), immune activation (Tellez et al., 2014), lameness (Wideman et al., 2014) and economic losses (Zuidhof et al., 2014). Removal of antibiotics from feed as growth enhancers that ameliorate intestinal inflammation further highlights the importance of studying IP in chickens

(Vicuna et al., 2015b). In addition to the limited literature on *in vivo* models to investigate increased IP in chicken, there is a dearth of knowledge regarding different IP biomarkers as reviewed by Gilani et al. (2016).

Fluorescein isothiocyanate dextran (FITC-d) is 3000–5000 Da molecular weight and does not traverse intestinal epithelial tight junctions in high quantities unless the intestinal barrier is compromised (Hamilton et al., 2015; Tan et al., 2015) and has been utilized in rat and mouse studies to evaluate increased IP. FITC-d has also been utilized in *in vitro* studies that employed Ussing chambers to measure increased IP in chickens (Song et al., 2013, 2014) and *in vivo* studies (Kuttappan et al., 2015; Vicuna et al., 2015b). Another similar biomarker used to measure increased IP is the leakage of lactulose (L), rhamnose (R) and mannitol (M) sugars through TJ. Lactulose with rhamnose or mannitol are used interchangeably. Lactulose being of higher molecular weight (342 Da) can traverse damaged TJ (paracellular pathway), while mannitol (182 Da) and rhamnose (164 Da) sugars can directly be absorbed through enterocytes (transcellular pathway) (Bjarnason et al., 1995; Hollander, 1999). Although this sugar method has been used extensively in rats, mice and humans (Bjarnason et al., 1995; Hollander, 1999; Tooley et al., 2009; Denno et al., 2014; Tran et al., 2015), this has not been previously investigated in chickens. Due to limited knowledge of this test in chickens, lactulose with rhamnose and mannitol were used together. Additionally, comparing this sugar method with the FITC-d technique will enhance our understanding of IP. The advantage of the lactulose, rhamnose and mannitol sugar test is that it is quite safe to use repeatedly in live chickens as it is routinely used in humans. Although there have not been any adverse reports of FITC-d use *in vivo*, it has not been employed routinely in humans.

Similarly, other biomarkers to evaluate IP changes in humans include diamine oxidase (DAO), D-lactate, intestinal fatty acid binding protein (IFABP) and α anti -trypsin inhibitor(AAT). DAO is a 250 000 Da enzyme that catalyses deamination of histamines (Wu et al., 2013) and its increased concentration in blood indicates increased IP (Sun et al., 2015; Xun et al., 2015). D-lactate is produced by gut microbes and can enter the circulation following the intestinal damage (Ni et al., 2015; Shi et al., 2015; Xun et al., 2015). Studies of DAO and D-lactate in chicken serum are limited (Lei et al., 2013; Wu et al., 2013) and require further research. IFABP is a 15 000 Da protein (Ni et al., 2015) that traverses TJs due to increased IP caused by intestinal necrosis or ischaemia in humans (He et al., 2014; Piton et al., 2015; Shi et al., 2015). In a recent study of increased IP in chickens, mRNA expression of IFABP-2 was decreased significantly (Chen et al., 2015). However, IFABP in serum or plasma of chickens has yet to be determined. AAT produced by the liver against intestinal inflammation can enter into the lumen due to intestinal damage and can be detected in faeces (Suchodolski et al., 2012; Parambeth et al., 2015). Although increased AAT levels in chicken serum have been observed following a coccidiosis challenge (Golab et al., 2007), its use in faeces has not been evaluated. In addition to the aforementioned biomarkers of increased IP in humans, a recent study has shown that TJ proteins can also be detected in diabetic human plasma due to increased IP (Jayashree et al., 2014). Although chicken-specific ELISA kits are available for TJ, there is no published literature on the use of this biomarker to study IP in chicken.

Dextran sodium sulphate is known to increase pro-inflammatory cytokines such as (TNFα, IL-10 and IL-12) leading to colon inflammation and damage to the TJ and causing increased IP ultimately. DSS has been extensively used as a colitis model for increased IP in rats (Howarth et al., 1998; Abimosleh et al., 2012; Yuan et al., 2015) and mice (Laroui et al., 2012; Perše and Cerar, 2012). DSS has been shown to cause intestinal inflammation, reduced villi height (Menconi et al., 2015) and increased IP in chickens (Kuttappan et al., 2015; Vicuna et al., 2015b). Similarly, fasting is known to reduce intestinal villi and cell area (Yamauchi and Tarachai, 2000) and increase mucosal damage (Thompson and Applegate, 2006) in chickens. Fasting has also been shown to increase IP in humans (Genton et al., 2015) and in chickens measured by increased FITC-d leakage (Kuttappan et al., 2015; Vicuna et al., 2015b) and bacterial translocation (Burkholder et al., 2008). We therefore hypothesized that increased IP in chickens could be detected by different biomarkers following DSS and fasting challenges. Specifically, we aimed to evaluate IP changes in blood by detection of lactulose, rhamnose and mannitol sugars in addition to FITC-d. Also AAT, DAO, D-lactate, IFABP and TJs tests were utilized.

Materials and methods

Study design

Forty-eight day-old male Ross 308 chicks were obtained from a local hatchery (Baiada, Gawler, Australia) and raised on indoor floor pens on wood shavings for 14 days. Birds were fed a commercially available broiler feed (Ridley Agri Products, Murray

Bridge, South Australia) and water *ad libitum*, with 16-h light: 8-h dark cycle. The birds were randomly allocated to three groups consisting of control, DSS treatment and 19.5-h fasting. They were then placed in metabolism cages (two birds per cage) for 2 days on day 14. After 2-day acclimatization, birds were transferred to individual metabolism cages. The DSS (MP Bio Medicals, NSW, Australia)-treated group received 0.75% DSS through their drinking water from day 16 until day 21. The fasting group was denied feed for 19.5 h before blood collection on day 21. All groups received *ad libitum* water.

All birds were monitored twice daily during rearing and throughout the study. Before shifting birds to the metabolic cages, all birds were weighed individually. Faecal trays of the metabolic cages were emptied daily in the morning for close observation of faecal consistency and discharge. The representative faecal samples were collected from the trays into a plastic container and then stored at −20 °C for further analysis.

Experimental protocol

All protocols were approved by the Animal Ethics Committees of the University of Adelaide and the Primary Industry Regions, South Australia. All animal studies were performed in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Oral solutions and gavages

Birds were orally gavaged with a feeding tube fitted to a disposable syringe. The tube had a rounded tip and was carefully inserted approximately 6–8 cm into the oesophagus. LMR was gavaged at 2 ml/bird (25 g lactulose, 5 g mannitol, 5 g rhamnose; Sigma-Aldrich NSW, Australia, dissolved in 100 ml milli-Q water 18.2 MΩ·cm at 25 °C). FITC-d (4000 mol weight; Sigma-Aldrich, NSW, Australia) was prepared at 2.2 mg/ml and was gavaged at 1 ml/bird. On day 21, all birds were given an oral gavage of FITC-d and 60 min later another oral gavage of LMR sugar solutions was administered.

Processing of blood samples

Blood samples (3 ml) were collected once per bird from the brachial vein 90 min following lactulose, rhamnose and mannitol oral gavage based on previous experiments (unpublished). Whole blood was divided into plasma or serum vacutainers (kept in ice and room temperature, respectively) (BD, NSW, Australia) and centrifuged at 1600 *g* for 10 min. The supernatant was collected, and plasma and serum samples were stored in 0.5 ml aliquots at −20 °C until further analysis.

High-performance ion chromatography (HPIC) analysis

Standards of lactulose, mannitol and rhamnose were prepared using fasted chicken plasma obtained from a local slaughterhouse and spiked to the following concentrations 125, 62.5 and 31.25 μM and stored at −20 °C until further analysis. Plasma and samples were thawed and were treated with 15% trichloroacetic acid (TCA; Sigma-Aldrich, Castle Hill, Australia) and centrifuged at 15 000 *g* for 10 min, and the supernatant was mixed with an equal volume of mixed-bed ion exchange matrix amberlite MB-1 resin (Sigma-Aldrich) for 30 min. The supernatant was transferred to Thomson single step™ 0.2-μm nylon filter vials (Adelab, Adelaide, Australia) and analysed using the Dionex ICS-4000 (ThermoFisher, Scoresby, Australia) HPIC system as described by (Tran et al., 2015). All samples were run in duplicate.

FITC-d analysis

FITC-d analysis was conducted as previously described (Vicuna et al., 2015b). Briefly, standards (0, 0.0001, 0.001, 0.01, 0.1, 1.0 and 10 μg/ml) were prepared using the same FITC-d sugars as for the oral gavage. Standards and serum samples were run in triplicate. The concentration of FITC-d in serum was calculated using a standard curve and a Synergy MX plate reader (Biotek Instruments, Bedfordshire, UK). The wavelengths for excitation and emission were 485 nm and 530 nm respectively.

IFABP, DAO, D-lactate, AAT and TJs ELISA tests

ELISA kits (chicken-specific antibodies) for IFABP, DAO, D-lactate, AAT and TJs were obtained from MyBioSource, (San Diego, USA). All standards and plasma samples (except for faecal extracts for AAT) were measured in duplicate in a microplate reader at 450-nm wavelength (Bio-Rad laboratories, California, USA) following manufacturer's instructions.

Statistical analysis

All statistical analyses were performed using SPSS 22 (IBM SPSS, Armonk, NY: IBM), and results were compared using one-way analysis of variance (ANOVA) with Tukey's *post hoc* test. Data from all results were

normally distributed except for the FITC-d (fasting) where the data were natural-log-transformed as variance between fasting and control groups was not equal. Statistical significance was considered if $p < 0.05$.

Results

Lactulose, rhamnose and mannitol sugars and their ratios

Plasma levels of lactulose, rhamnose and mannitol in μ mol/l are shown in Table 1, while L/R and L/M ratios are shown in Fig. 1. Average plasma lactulose concentration was significantly higher ($p < 0.05$) for fasting and DSS compared with control (5.71, 5.29 and 3.64 μ mol/l, respectively), but did not differ statistically between fasting and DSS models. Average rhamnose concentration for DSS treatment was significantly higher ($p < 0.05$) compared with control and fasting (19.37 vs. 15.28 and 16.56 μ mol/l respectively). Fasting did not increase rhamnose levels compared with control. In contrast, mannitol concentration in fasted chicken was significantly lower ($p < 0.05$) compared with control and DSS groups (16.49, 21.19 and 23.24 μ mol/ml respectively). L/R and L/M ratios were significantly higher ($p < 0.05$) for fasting and DSS treatment compared with control ($0.35 > 0.28 > 0.24$ for L/R and $0.35 > 0.23 > 0.18$ for L/M) as shown in Fig. 1.

Different biomarkers for assessing IP

Figure 2 shows FITC-d concentration in fasted chicken was significantly higher compared with DSS and control groups. Table 2 shows five biomarkers that were used in the current study to assess IP. D-lactate concentration for fasting treatment was significantly lower than control, but DSS did not significantly reduce D-lactate. TJ concentration in blood showed significantly higher concentration for fasting compared with control, but for DSS treatment concentration was not significantly different.

Table 1 Mean ± Standard error values of individual sugars (μ mol/l) in chicken plasma ($n = 16$) following DSS and fasting treatment

Treatment	Lactulose	Rhamnose	Mannitol
Control	3.64 ± 0.11^{a}	15.28 ± 0.77^{a}	21.19 ± 1.0^{a}
DSS	5.29 ± 0.17^{b}	19.37 ± 0.50^{b}	23.24 ± 0.89^{a}
Fast	5.71 ± 0.25^{b}	16.56 ± 0.80^{a}	16.49 ± 0.67^{b}

Different superscripts indicate significant differences ($p < 0.05$) within each column.

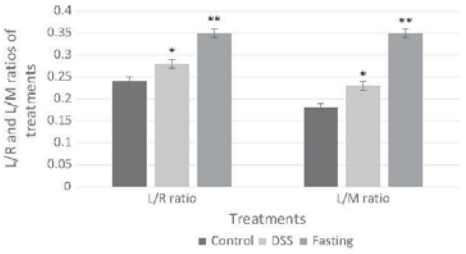

Fig. 1 Lactulose to rhamnose (L/R) and mannitol (L/M) ratio ($n = 16$) for control, DSS and fasting * and ** denote statistical significance at $p < 0.05$ compared with control and DSS treatments respectively.

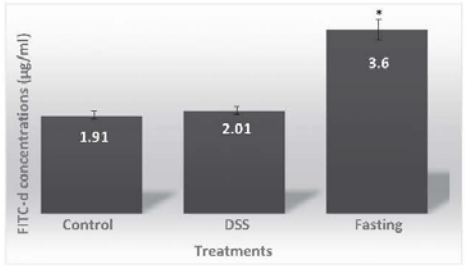

Fig. 2 FITC-d concentration (μg/ml) in serum of control ($n = 16$), DSS ($n = 16$) and fast ($n = 13$) * denotes statistical significance at $p < 0.05$ compared to control (Three samples in fasting group could not be used due to severe haemolysis during blood collection).

Discussion

The aim of the current study was to compare L/M, L/R, FITC-d sugar permeability tests, IFABP, DAO, D-lactate and TJs biomarkers to evaluate increased IP in chickens *in vivo* using DSS and fasting models as these have been shown to increase IP in chickens (Kuttappan et al., 2015; Vicuna et al., 2015b). Limited literature regarding biomarkers available to evaluate IP in chickens *in vivo* prompted the current study. Although the lactulose, rhamnose and mannitol sugar test has been used in other species, it has not been evaluated in chickens nor compared with the FITC-d test that has recently been utilized in chickens.

It is problematic with lactulose, rhamnose and mannitol sugars (if measured individually) that passage, and complete removal from the digestive system can be affected by the presence of food material. To overcome this problem, the concept of L/M and L/R ratio was introduced (Hollander, 1999). Ideally, increased

Table 2 Mean ± Standard error concentrations (n = 10) of five different tests for increased intestinal permeability of chickens

Test	Treatment	Mean ± SE
IFABP (ng/ml)	Control	$0.60^{a} \pm 0.09$
	DSS	$0.49^{a} \pm 0.03$
	Fast	$0.41^{a} \pm 0.02$
DAO (ng/ml)	Control	$12.71^{a} \pm 0.72$
	DSS	$12.75^{a} \pm 1.31$
	Fast	$14.57^{a} \pm 1.49$
D-lactate (μg/ml)	Control	$4.04^{a} \pm 0.22$
	DSS	$3.67^{a} \pm 0.20$
	Fast	$2.57^{b} \pm 0.17$
AAT (μg/ml)	Control	$1.61^{a} \pm 0.19$
	DSS	$1.52^{a} \pm 0.24$
	Fast	$1.57^{a} \pm 0.06$
TJ (pg/ml)	Control	$643.6^{a} \pm 0.04$
	DSS	$555.1^{a} \pm 0.02$
	Fast	$1025.6^{b} \pm 0.05$

Different superscripts denote statistical significance at $p < 0.05$ within each test.

L/M or L/R ratios due to increased lactulose and either reduced or similar rhamnose and mannitol concentrations would indicate increased IP (Bjarnason et al., 1995). The current study has shown that fasting increased lactulose, while mannitol was decreased. At the same time, rhamnose concentration was not statistically different than control, indicating that fasting increased IP, in agreement with above principle and previous studies of these sugars in different species (van Wijck et al., 2013; Denno et al., 2014; Araujo et al., 2015; Shaikh et al., 2015; Tran et al., 2015; Wang et al., 2015). DSS showed a different pattern for L/M and L/R ratios (Fig. 1). These results are against the principle of the L/R ratio; however, the findings for the L/M ratio are in agreement with previous studies as mentioned above. L/R and L/M have not been investigated together, which does not give clear indication why rhamnose absorption was increased. In conclusion, the L/M ratio appeared more sensitive than the L/R ratio at detecting IP increases in chickens. As fasting and DSS ingestion increase IP by different mechanisms, further confirmatory studies are required to identify the L/M sugar ratio as an optimal biomarker for intestinal permeability changes in chickens.

The FITC-d results revealed that fasting significantly increased IP compared with the control and DSS methods (Fig. 2). Interestingly, both Kuttappan et al. (2015) and Vicuna et al. (2015b) also reported that 24–29 h of fasting increased IP in 3-day-old chicks compared with non-fasted chicks. In the current study, chickens at 21 days of age were fasted for 19.5 h resulting in increased IP as measured by FITC-d. The average FITC-d concentration for fasting (3.6 μg/ml) compared with control concentration (1.91 μg/ml) was higher than that of a previous study (Vicuna et al., 2015a). This discrepancy could have been due to light exposure of FITC-d during preparation of standards. However, on a relative basis the FITC-d results (control vs. fasting) were comparable to previous studies. Fasting increased the concentration of FITC-d by 1.8 times compared with the concentration of the control group. Contrary to previous reports (Kuttappan et al., 2015; Vicuna et al., 2015b), DSS did not increase IP measured by FITC-d. In the current study, DSS was administered in the drinking water following the method reported by Vicuna et al. (2015b). Discrepancies between the studies could therefore have been due to different strains of chickens used in these studies. Vicuna et al. (2015b) used Cobb, whereas Ross 308 was used in the current study. It is known that DSS can affect IP differently in different strains as reviewed by Perše and Cerar (2012). Additionally, age may also have had an effect; however, it has not been cited.

Lactulose and mannitol sugar results showed that DSS increased IP, suggesting that the LMR sugar test might be more sensitive than the FITC-d test. It is possible that the DSS treatment did not damage the TJs to an extent that it would have increased FITC-d leakage, but the disruption was enough to increase lactulose concentration in blood. This premise is supported by the fact that lactulose is a smaller sugar (342 Da) (Bjarnason et al., 1995) compared to FITC-d 3000–5000 Da (Hamilton et al., 2015). Figure 3 compares the molecular weights of FITC-d and DAO, suggesting

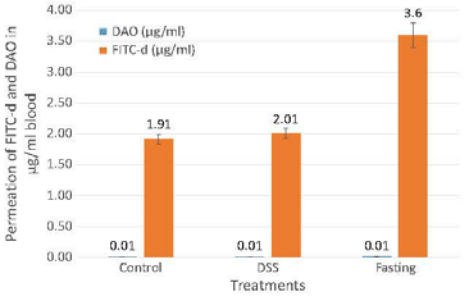

Fig. 3 Passage of FITC-d (4000 Da) and DAO (250 000 Da) into blood from the intestine showing higher the molecular size, lower the passage.

that molecules of greater molecular weight had lower passage from the intestine into plasma. Additionally, fasting and DSS treatments have been applied in chickens (Kuttappan et al., 2015), where both treatments increased IP as measured by FITC-d and their effects were similar to each other. However, in the current study, there was greater translocation of FITC-d across the intestinal epithelium in fasted chickens than in DSS-treated or control birds.

The decrease in IFABP concentration for DSS and fasting treatments in the current study was inconsistent with published human studies where increased IP was linked with increased IFABP due to intestinal ischaemia (He et al., 2014; Ni et al., 2015; Piton et al., 2015; Shi et al., 2015). One recent paper suggested that DSS treatment in mice decreased fatty acid absorption (Henderson et al., 2015). This may explain why DSS treatment decreased IFABP concentration in chickens, as the purpose of IFABP is to transport fatty acids. With respect to fasting, it may have been that fasting and ischaemia did not increase IP through similar mechanisms. Additionally, the molecular size of IFABP is 15 000 Da (Ni et al., 2015), which is much higher than FITC-d (3000–5000 Da) and lactulose (342 Da), suggesting that DSS and fasting may not have damaged intestinal TJ to such an extent that it would allow 15 000 Da protein to pass through the intestinal epithelium (Fig. 3). This was consistent with our observation that DSS or fasting did not produce blood-stained diarrhoea or mortality. In a separate study in piglets, unlike citrulline, IFABP levels did not increase after a short fasting period (3 h) in a weaning stress model (Berkeveld et al., 2008) indicating that IFABP may not be a reliable biomarker of increased IP in pigs.

Diamine oxidase concentration results are in contrast with other studies in chickens in which increased DAO concentrations were associated with increased IP (Lei et al., 2013; Wu et al., 2013; Li et al., 2015). However, in these studies LPS treatment was used compared with DSS ingestion and fasting in the current study. Only two studies in chickens have used DSS and fasting to increase IP; however, DAO was not used in either of these studies (Kuttappan et al., 2015; Vicuna et al., 2015b). One study in chickens revealed that *S. pullorum* inoculation did not increase DAO levels, suggesting that mild inflammation may not increase DAO concentration (Wang et al., 2012). One study in rats showed that DAO concentration was not increased due to DSS treatment. They showed that the small intestine tissue was not damaged and the nutrient absorption was not changed (Sakata et al., 2011). Another possible reason could have been the degree of damage to TJs and the comparative greater molecular size of DAO (250 000 Da) (Wu et al., 2013), much higher than lactulose and FITC-D sugars as explained above. Moreover, Sakata et al. (2011) reported that nutrients can also impact on DAO release. It has been reported that fasting (duration not mentioned) increased DAO activity in the intestine (Bamba et al., 1990; Erdman, 1990; Zhou et al., 2011). The current study therefore represents the first to measure DAO in chickens subjected to fasting as a model for increased IP.

D-lactate has been used in chickens; however, the current study is the first to measure D-lactate following DSS and fasting treatments. The D-lactate values did not indicate IP increase. DSS treatment is used as a colitis model (inflammation of the large intestine) in rats and mice and this may alter lactic acid-producing bacteria populations, reducing D-lactate production. Although no direct studies have investigated this effect, a study by Rogala et al. (2015) revealed that the intensity of DSS-related inflammation in mice was affected by different microbial communities, suggesting that the converse may also be possible. Fasting, however, significantly reduced D-lactate concentration in the current study. This is in contrast to a study in which fasting showed an increased D-lactate concentration in mice (Kondoh et al., 1992); however, D-lactate in the Kondoh et al. (1992) study was measured in urine compared with plasma in the current study. Fasting has been linked to altered intestinal microbiota in quails (Kohl et al., 2014), suggesting this may be similar with chickens possibly leading to reduced D-lactate concentration in plasma consistent with the current study. Our results therefore suggest that D-lactate may not be a suitable biomarker for studying IP in chicken using DSS and fasting models.

α Anti -trypsin inhibitor concentration results of the current study are in contrast to previous studies in humans (Suchodolski et al., 2012; Parambeth et al., 2015). Although increased AAT concentration in plasma was observed in chickens (Golab et al., 2007), the coccidiosis model used might have been quite severe compared with DSS and fasting. It is known that severity of intestinal inflammation is directly related to AAT leakage in faeces (Suchodolski et al., 2012). AAT may therefore be a useful biomarker in models associated with greater intestinal damage.

ZO-1 has an important role in regulating intestinal permeability and is localized in tight junctions (Fink, 2003). Increased plasma ZO-1 concentration has been observed in coeliac disease, diabetic and insulin-resistant human patients (Klaus et al., 2013) and rats (Jayashree et al., 2014). The results of the current

study revealed that fasting increased plasma TJs significantly ($p < 0.05$) but not for DSS treatment. As discussed above, fasting could have been more effective at increasing IP than the DSS treatment. ZO-1 biomarker has been recently developed and there are very few studies in human and rats. Additionally, our previous studies (unpublished data) have shown variation between different ELISA kits; hence, this test may need further investigation.

Conclusions

The current study investigated different biomarkers to assess IP in chickens. DSS ingestion and fasting were used as independent models to increase IP. IFABP, DAO, D-lactate and AAT concentrations did not reveal an association with increased IP in these models. Fasting significantly increased IP measured by FITC-d in addition to lactulose, rhamnose and mannitol sugar concentrations in blood. The DSS ingestion model only showed increased IP by the lactulose to mannitol ratio. This suggests that the lactulose to mannitol ratio may be a more sensitive method to assess altered IP in chickens compared with FITC-d.

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Chapter 5: Reduced fasting periods increase intestinal permeability in chickens

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SHORT COMMUNICATION

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Reduced fasting periods increase intestinal permeability in chickens

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Summary

Fasting of up to 24 hr has been shown to increase intestinal permeability (IP) in chickens. The aim of this study was to determine whether fasting duration of 4.5 and 9 hr increased IP and whether L-glutamine (a non-essential amino acid) supplementation before fasting provided some protection of barrier function as shown in other species. Ross 308 male broilers ($n = 96$) were fed either a control diet or the same diet supplemented with 1% glutamine from d0 to d38 post-hatch. On d37, the birds were assigned to single-bird metabolism cages and were fasted for either 0, 4.5, 9 or 19.5 hr. This study design was 2 × 4 factorial with two levels of glutamine and four levels of fasting. Birds in the 0-hr fasting group had free access to feed. All birds had ad libitum access to water. To measure IP on day 38, following their respective fasting periods, birds were administered two separate oral gavages of fluorescein isothiocyanate dextran (FITC-d) followed by lactulose, mannitol and rhamnose (LMR) sugars, 60 min apart. Whole blood was collected from the jugular vein 90 min post-LMR sugar gavage. FITC-d and L/M/R ratios were measured by spectrophotometry and high-performance ionic chromatography respectively. Lipopolysaccharide (LPS) endotoxins in plasma of the birds fed the control diet were also measured using chicken-specific LPS antibody ELISA. Serum FITC-d and plasma L/M and L/R ratios for 4.5, 9 and 19.5 hr were significantly ($p < .05$) higher compared to the non-fasting group. However, IP was not different in the glutamine-supplemented group ($p > .05$) compared to the control group. LPS concentrations measured by the ELISA were below the detectable range. We conclude that fasting periods of 4.5 and 9 hr increased IP compared to non-fasted birds and dietary glutamine supplementation did not ameliorate changes in IP.

KEYWORDS

fluorescein isothiocyanate dextran, glutamine, lipopolysaccharides, feed withdrawal and sugar ratio

1 | INTRODUCTION

Feed restriction is a common practice in chicken meat production where birds are usually fasted prior to slaughter to minimize carcass contamination with intestinal contents during processing. During the depopulation process, some birds in the same shed are often deprived of feed for up to 12 hr (Allen et al., 2008; Delezie, Swennen, Buyse, & Decuypere, 2007). Additionally, broiler

breeders are often deprived of feed for weight management and production (Najafi, Zulkifli, Soleimani, & Kashiani, 2015). Fasting for periods of up to 24 hr has been shown to increase stress measured by increased corticosterone levels (Najafi, Zulkifli, Soleimani, & Goh, 2016) and affect intestinal morphology (reduced crypt depth and increased mucin production; Burkholder, Thompson, Einstein, Applegate, & Patterson, 2008; Thompson & Applegate, 2006). Studies have shown a reduction in villus height due to fasting, possibly due to reduced cell proliferation and migration rates (Ferraris & Carey, 2000). Fasting during thinning in poultry productions also increased in vivo risk of *Campylobacter* colonization (Allen et al., 2008) and ex vivo attachment of *Salmonella enteritidis* in the ileum (Burkholder et al., 2008) and can potentially be harmful for human health and food safety. Recently, fasting for 19.5 hr in 21-day-old broiler chickens (Gilani et al., 2016a) and 24 hr in 7 days old broiler chickens (Kuttappan et al., 2015; Vicuna et al., 2015) has been shown to increase intestinal permeability (IP). Increased IP is the increased passage of intestinal contents from the lumen to blood and may lead to reduced performance and compromised health (Gilani, Howarth, Kitessa, Forder, et al., 2016). However, the effects of short-term fasting periods of 4.5 and 9 hr have yet to be investigated.

Glutamine is a non-essential amino acid, deposited in skeletal muscle (Stachowicz-Stencel & Synakiewicz, 2012), and provides a substrate for intestinal cell proliferation. Studies have shown that glutamine improves IP in in vitro human cell cultures (Le Bacquer, Laboisse, & Darmaun, 2003), in a weaning stress model in pigs (Wang, Zhang, et al., 2015) and a mucositis model in rats (Beutheu et al., 2014) by regulating tight junction protein expression as reviewed by Wang, Wu et al. (2015). Limited in vitro studies in chickens have also shown that glutamine improves barrier integrity (Awad & Zentek, 2015; Awad et al., 2015). In the aforementioned studies, glutamine was administered post-challenge, while glutamine as a preventative treatment has also been utilized as reviewed by (Andrade et al., 2015; Wang, Wu, et al., 2015). In brief, glutamine has been shown to maintain IP in mice when fed before a heat stress challenge (Soares et al., 2014). Similarly, feeding glutamine before an exercise and intestinal ischaemia challenge maintained IP in humans (Zuhl et al., 2014) and rats (Wang, Niu, et al., 2015). However, the role of glutamine in gut permeability in chickens has yet to be explored. There are a few published scientific papers regarding biomarkers of increased IP in chickens. Fluorescein isothiocyanate dextran (FITC-d) has recently been utilized as permeability marker in chickens (Kuttappan et al., 2015; Vicuna et al., 2015), and we have recently utilized FITC-d and lactulose, rhamnose and mannitol sugar methods (Gilani et al., 2016a). Both methods were utilized and compared in the current study.

The main objectives of this study were to investigate whether reduced fasting periods of 4.5, 9 and 19.5 hr increased intestinal permeability as measured by two different methods (lactulose/rhamnose/mannitol ratio and the fluorescein isothiocyanate dextran (FITC-d) test), and to determine whether glutamine supplementation before fasting at 38 days of age ameliorated increased IP in chickens.

2 | MATERIALS AND METHODS

2.1 | Animals and diets

Ross 308 male chickens (n = 96) were reared in raised floor pens with wood shavings as bedding material and were either fed a control (basal) diet or a diet supplemented with glutamine (1% in basal diet). Floor pens were 180 × 120 cm with 10 birds per pen reared under infrared heater lamps (25–30°C). All birds were given ad libitum feed and water with fourteen hours of light. The basal diet (wheat, sorghum and soya based) was formulated to meet the nutrient requirements of Ross 308 (Table 1). L-Alanine (1.22%) was added to the control diets to balance the nitrogen content with the glutamine-supplemented diets. Chicken starter, grower and finisher diets were fed during 0–9, 10–23 and 24–38 days of age respectively. On day 35, all birds were transferred in pairs to metabolism group cages (57 × 48 × 38 cm). After two days of adaptation, body weights were measured and chickens were allocated to individual cages and assigned to four fasting treatments: 0, 4.5, 9 and 19.5 hr (n = 12 per treatment per diet group).

All protocols were approved by the Animal Ethics Committees of the University of Adelaide and the Primary Industries and Regions South Australia (PIRSA). All animal studies were performed in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2 | Oral solutions and gavages

Following the fasting periods on day 38, all birds were given an oral gavage of 1 ml FITC-d (4,000 mol weight; Sigma-Aldrich, NSW, Australia) containing 2.2 mg dissolved in one ml Milli-Q water 18.2 MΩ cm at 25°C. Sixty minutes later, each bird received 2 ml of second gavage solution comprised of lactulose, mannitol and rhamnose (LMR). LMR solution contained 25 g lactulose, 5 g mannitol, 5 g rhamnose; Sigma Aldrich, and dissolved in 100 ml Milli-Q water 18.2 MΩ cm at 25°C.

2.3 | Processing of blood samples

A blood sample was collected from the jugular vein at 90 min post-lactulose, rhamnose and mannitol oral gavage. Each whole blood sample was divided into two aliquots for preparation of plasma and serum in vacutainers (BD, NSW, Australia).Each aliquot was centrifuged at 1,600 g for 10 min. The supernatants were collected and stored in 0.5 ml aliquots at −20°C until further analysis.

2.4 | High-performance ion chromatography (HPIC) analysis

Standards and samples were prepared as previously described by Gilani et al. (2016a); Gilani et al. (2016b) with some modifications. Lactulose (1.28 mg), mannitol (0.68 mg) and rhamnose (0.68 mg) were each mixed with 30 ml fasted human plasma, to prepare standard

TABLE 1 Composition of control and glutamine-supplemented diets (as formulated)

	Starter		Grower		Finisher	
Raw materials %	Control	Glutamine	Control	Glutamine	Control	Glutamine
Wheat	32.5	32.5	36.7	36.7	42.0	42.0
Soybean meal	31.7	31.7	26.7	26.7	23.0	23.0
Sorghum	20.0	20.0	20.0	20.0	20.0	20.0
Canola meal expeller	5.0	5.0	6.0	6.0	4.8	4.8
Canola oil	5.0	5.0	5.0	5.0	5.4	5.4
Di-calcium phosphate	1.8	1.8	1.6	1.6	1.4	1.4
Limestone	1.2	1.0	1.1	1.1	1.0	1.0
L-Alanine	1.2	0.0	1.2	0.0	1.2	0.0
L-Glutamine	0.0	1.0	0.0	1.0	0.0	1.0
Sodium bicarbonate	0.4	0.4	0.4	0.4	0.4	0.4
DL-Methionine	0.4	0.4	0.3	0.3	0.3	0.3
L-Lysine	0.3	0.3	0.3	0.3	0.2	0.2
L-Threonine	0.2	0.2	0.2	0.2	0.1	0.1
Premix + xylanase	0.2	0.2	0.2	0.2	0.2	0.2
Salt	0.1	0.1	0.1	0.1	0.1	0.1
Zinc oxide	0.02	0.02	0.02	0.02	0.02	0.02
Choline chloride	0.06	0.06	0.08	0.08	0.08	0.08
Sand	0.005	0.23	0.21	0.42	0.005	0.22
Nutrients %						
Dry matter	88.8		88.7		88.4	
ME MJ/kg	12.6		13.0		13.0	
Crude protein	22.6		20.7		19.5	
Crude fat	6.8		7.9		7.2	
Crude fibre	2.6		2.6		2.6	
Ash	5.9		5.6		4.9	
Digestible lysine	1.3		1.2		1.0	
Digestible methionine	0.7		0.6		0.5	
Digestible cysteine	0.3		0.3		0.3	
Digestible threonine	0.8		0.8		0.7	
Digestible tryptophan	0.3		0.2		0.2	
Calcium	0.9		0.9		0.8	
Phosphorus available	0.5		0.4		0.4	

Composition of the premix per kg of diet; vit. A 14,000 IU, vit. D_3 5,000 IU, vit. E 75 mg, vit. K_3 3.75 mg, vit. B_1 3 mg, vit. B_2 9 mg, vit. B_6 5 mg, vit. B_{12} 0.03 mg, biotin 0.2 mg, pantothenic acid 15 mg, folic acid 2.5 mg, niacin 55 mg, copper 20 mg, cobalt 0.25 mg, iodine 1.25 mg, iron 40 mg, manganese 120 mg, molybdenum 2 mg, selenium 0.3 mg, phytase 100 mg, ethoxyquin 100 mg.

solutions with concentrations of 125, 62.5 and 31.25 μM for each sugar. These standards were stored at −20°C until further analysis to minimize experimental standards error. Standards and samples were thawed and treated with acetonitrile (2:1 acid to standard or sample ratio; Sigma-Aldrich, Castle Hill, NSW, Australia), vortexed for 30 s and then centrifuged at 15,000 *g* for 20 min. The supernatant was transferred using syringe filters nylon 13 mm 0.22 μm (VWR International Pty, Brisbane, Qld, Australia) and analysed using a Dionex ICS-4000 (ThermoFisher Pty, Scoresby, Vic., Australia) HPIC system as described by Tran et al. (2015).

2.5 | FITC-d analysis

Standards and samples were prepared as previously described by Gilani et al. (2016a); Gilani et al. (2016b). Briefly, standards (0, 0.0001, 0.001, 0.01, 0.1, 1.0 and 10 μg/ml) were prepared using the same

FITC-d as for the oral gavage. Standards and serum samples were run in duplicate. The concentration of FITC-d in serum was calculated using a standard curve and a Synergy MX plate reader (Biotek Instruments, Bedfordshire, UK). The wavelengths for excitation and emission were 485 and 530 nm respectively.

2.6 | Enzyme-linked immunosorbent assay of lipopolysaccharide (LPS)

Chicken-specific LPS antibody ELISA kit was obtained from MyBioSource (San Diego, CA, USA). Standards and plasma samples were measured in duplicate in a microplate reader at 450-nm wavelength (Bio-Rad laboratories, CA, USA) following manufacturer's instructions.

2.7 | Statistical analysis

All statistical analyses were performed using SPSS 22 (IBM SPSS, Armonk, NY: IBM). Levene's test and Shapiro-Wilk tests (with Q–Q plot) were utilized for checking the variances and normality of the data respectively. Results were compared using generalized linear model (GLM) univariate with Tukey's and Tamhane's T2 post hoc test. The interaction between glutamine-supplemented and non-supplemented diet was also measured. A GLM multivariate model was also used to investigate the interaction of body weights with fasting and diet treatments. The ELISA results for LPS for 0–19.5 hr fasted groups from control diet groups were analysed by using one-way analysis of variance (ANOVA). Data from lactulose, rhamnose, mannitol sugars and LPS were normally distributed. However, the data for the FITC-d did not have equal variance and were natural-log-transformed. Statistical significance was considered at $p < .05$.

3 | RESULTS

3.1 | Body weights

Mean body weights of all treatments are shown in Table 2. Body weights prior to fasting between all groups were not significantly different ($p = .998$). The interactions between body weight, diets and fasting treatments were also not statistically different ($p = .89$).

3.2 | FITC-d

Serum FITC-d concentrations (μg/ml) were significantly increased with increasing fasting time ($p = .001$), irrespective of glutamine treatment (Table 2). Without glutamine supplementation, mean serum FITC-d concentration for 0, 4.5, 9.0 and 19.5 hr of feed withdrawal was 0.94, 1.25, 1.69 and 1.61 μg/ml respectively. With glutamine supplementation, mean FITC-d concentrations in serum for the above four feed withdrawal periods were 0.95, 1.38, 1.69 and 1.74 μg/ml respectively. Serum FITC-d concentrations between 9 and 19.5 hr did not differ statistically ($p = .69$), but were significantly ($p = .03$) higher than the FITC-d concentrations at 0- and 4-hr fasting.

TABLE 2 Body weights, individual sugars (μmol/L), lactulose/mannitol ratio, lactulose/rhamnose ratio and FITC-d (μg/ml) ± standard error concentrations

Fasting hours	Control							Glutamine supplemented						
	BW	Lactulose	Rhamnose	Mannitol	L/M	L/R	FITC-d	BW	Lactulose	Rhamnose	Mannitol	L/M	L/R	FITC-d
0	3097.08 ± 42.18	3.60 ± 0.10^a	9.61 ± 0.26	15.38 ± 0.59	0.24 ± 0.01^a	0.38 ± 0.02^a	0.94 ± 0.03^a	3206.58 ± 51.07	3.40 ± 0.19^a	9.15 ± 0.34	16.37 ± 1.80	0.24 ± 0.03^a	0.38 ± 0.02^a	0.95 ± 0.07^a
4.5	3038.83 ± 41.49	5.10 ± 0.23^b	9.84 ± 0.30	13.93 ± 1.39	0.39 ± 0.03^b	0.52 ± 0.02^b	1.25 ± 0.07^b	3043.25 ± 40.36	4.86 ± 0.27^b	8.77 ± 0.17	12.36 ± 0.70	0.41 ± 0.04^b	0.56 ± 0.03^b	1.38 ± 0.08^b
9.0	3121.17 ± 86.05	5.17 ± 0.19^b	9.43 ± 0.74	13.96 ± 1.22	0.40 ± 0.04^b	0.59 ± 0.06^b	1.69 ± 0.06^c	3050.41 ± 73.99	4.72 ± 0.19^b	9.05 ± 0.40	11.82 ± 0.72	0.41 ± 0.02^b	0.53 ± 0.03^b	1.69 ± 0.05^c
19.5	3025.50 ± 65.19	5.08 ± 0.18^b	9.33 ± 0.45	12.92 ± 1.13	0.43 ± 0.04^b	0.56 ± 0.02^b	1.61 ± 0.11^c	3019.41 ± 91.38	5.20 ± 0.31^b	9.49 ± 0.37	13.81 ± 0.93	0.40 ± 0.04^b	0.55 ± 0.03^b	1.74 ± 0.07^c

Different superscripts show the significance between the treatments at $p < .05$.

3.3 | Lactulose, rhamnose, mannitol sugars concentration and ratios of L/M and L/R

Individual sugar concentrations in plasma (μmol/L) of lactulose, rhamnose and mannitol and their ratios (L/M and L/R) are shown in Table 2. There was no interaction between treatments and fasting times (p = .57). Mean L/M and L/R ratio increased significantly for 4.5, 9 and 19.5 hr in the fasted groups compared to non-fasted (p = .001). However, these ratios were not statistically different between different fasting times (p = 1.00). There was no significant difference between un-supplemented and glutamine-supplemented groups (p = .70).

3.4 | LPS ELISA results

The concentrations of LPS from only non-supplemented groups that were fasted for 0, 4.5, 9 and 19.5 hr were 0.023 μmol/L (data not shown). Because glutamine fed groups did not show any significant differences, these groups were not analysed using ELISA. The concentrations of LPS were below the detection limit, and hence, no statistical difference was found between different fasted groups (p = .55).

4 | DISCUSSION

The aim of this study was to investigate the effects of different fasting periods on IP and whether dietary glutamine supplementation could restore gut barrier function. Fasting for 19.5 and 24 hr has been shown to increase IP in chickens (Gilani et al., 2016a; Vicuna et al., 2015). Fasting is also applied during complete or partial depopulation of flocks prior to slaughter.

There was no interaction between bodyweight and lactulose, rhamnose and mannitol sugars tests (p = .57), and also no interaction between bodyweight and fasting (p = 1.0). Similar body weights between glutamine-supplemented and non-supplemented groups may have been due to the fact that both diets were iso-caloric and iso-nitrogenous. These results are consistent with previous studies in chickens in which glutamine supplementation did not change body weights significantly at day 21 or beyond (Bartell & Batal, 2007; Murakami, Sakamoto, Natali, Souza, & Franco, 2007).

In the present study, we demonstrated that fasting, as early as 4.5 hr, significantly increased IP in chickens, as measured by FITC-d. The difference in IP was further increased at 9 hr and then did not increase further for 19.5 hr of fasting. Serum FITC-d concentrations for 4.5, 9 and 19.5 hr of fasting were 1.3, 1.8 and 1.7 times higher compared to control. Increased IP for 19.5 hr fasting was comparable to previous studies (Gilani et al., 2016a) and (Kuttappan et al., 2015; Vicuna et al., 2015) where fasting for 19.5–24 hr increased serum FITC-d concentration by up to twofold, compared to non-fasted groups.

Due to limited published literature regarding the biomarkers for measuring in chickens, lactulose-to-mannitol (L/M) and lactulose-to-rhamnose (L/R) ratio markers were also applied in this study. Similar to the FITC-d data, L/M and L/R ratios for 19.5 hr of fasting were comparable to those in a previous study (Gilani et al., 2016a). The absolute values for non-fasted and fasted groups in the current study were slightly higher than previously reported (Gilani et al., 2016a) and may have been due to slight changes in the HPIC protocol (acetonitrile acid vs. tricarboxylic acid (TCA) in the previous study). TCA and acetonitrile were used to precipitate proteins in plasma samples to allow sugar levels to be determined through HPIC. The ratios for L/M and L/R for 4.5 and 9 hr fasting groups increased compared to the non-fasted group. Although these ratios were lower than the ratio for the 19.5 hr fasted group (1.8 times), this difference was not statistically significant. Some researchers have argued that individual sugar values should be examined during the L/M and L/R ratio investigation (Araujo et al., 2015; Shaikh, Rajan, Forsyth, Voigt, & Keshavarzian, 2015). In the current study, lactulose concentrations increased significantly with increasing fasting periods. Lactulose passes through the paracellular pathway, while rhamnose and mannitol pass through the transcellular pathway as reviewed by Bjarnason, Macpherson, and Hollander (1995). Rhamnose and mannitol concentrations were similar between all groups. These observations are consistent with the basic principle of L/M and L/R ratio (Bjarnason et al., 1995) that increased lactulose with similar rhamnose and mannitol values should be observed in increased IP. Finally, FITC-d, lactulose, L/M and L/R ratio were not highly correlated with each other in the current study (<.5 correlation coefficient).

Although depopulation (fasting for 24 hr) has been shown to increase bacterial translocation in chickens (Burkholder et al., 2008) in vitro however, in the current study, the significant increment in IP evident after 4.5 and 9 hr fasting is noteworthy, as increased IP may lead to increased bacterial translocation. No previous studies have been conducted in chickens over serial fasting time periods with regard to effects on increasing IP. Additionally, in the current study, the sugar methods were applied in vivo. The deleterious effects of short-term fasting and increased bacterial translocation remain to be investigated.

The observations on glutamine supplementation in our study were contrary to previous studies in which prophylactic use of glutamine has been shown to maintain intestinal IP in rats and humans (Mondello et al., 2010; Soares et al., 2014; Wang, Niu, et al., 2015; Zuhl et al., 2014). Experimental models, animal species and the severity of these models to increase IP may explain the difference in results for glutamine supplementation in maintaining IP. Although glutamine is found in abundance in blood, skeletal muscles and other fluids, its requirement increases during stress (Wang, Wu, et al., 2015) and fasting of in vitro cell culture (Le Bacquer et al., 2003). Also, it has been suggested that during pathological conditions and stress the release of glutamine may be insufficient to meet requirements (Stachowicz-Stencel & Synakiewicz, 2012). This may explain why glutamine fed chickens failed to maintain intestinal IP during the current fasting challenge. Other potential explanations could be that the glutamine dose used (10 g/kg) was insufficient to ameliorate increased IP effects. Further studies are needed to establish the effects of glutamine following a challenge, particularly fasting.

In addition to dual sugar methods, lipopolysaccharide (LPS) uptake has been utilized recently to evaluate increased IP changes in chickens (Chen, Tellez, Richards, & Escobar, 2015). LPS may provide an alternative to sugar markers as this does not require oral gavage and the ELISA kit is commercially available. LPS is produced from gram negative bacteria and can pass through tight junctions in increased IP in chickens (Chen et al., 2015) and has been utilized in human studies recently (Jayashree et al., 2014; McDonald et al., 2016). LPS concentrations in this study were lower than the detection range of the ELISA kit and hence were not statistically different. The absolute values in this study (were 0.023 μmol/L) were also different compared to a previous study in chickens (159 pg/ml; Chen et al., 2015). A different model (high dosage of coccidiosis vaccine vs. fasting in this study) may have been a reason for this. Fasting for 2–7 hr has also been shown to alter the phylogenetic diversity of microbiota in quails (Kohl, Amaya, Passement, Dearing, & McCue, 2014). Because LPS is produced predominantly from the microbiota, this may explain the lower and static LPS concentration across all fasted groups of control diet fed group suggesting that LPS may not be an appropriate marker of IP in fasting challenge.

5 | CONCLUSIONS

Short fasting periods of 4.5 and 9 hr increased IP as measured by FITC-d and, L/M and L/R ratio markers. However, prophylactic feeding of glutamine did not improve IP during fasting. Further studies are necessary to investigate the optimal fasting period to avoid increased IP in chickens prior to slaughter. Once determined, this would have the potential to prevent chicken meat contamination. Also further studies are necessary to investigate whether varying concentrations of dietary glutamine supplementation after a fasting challenge improves IP.

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Chapter 6: Effects of delayed feeding, sodium butyrate and glutamine on intestinal permeability in newly-hatched broiler chickens

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Contribution to the Paper	Performed literature search, planned and conducted the experiment, analysed the blood samples and data, wrote the manuscript and acted as corresponding author		
Overall percentage (%)	75		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

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SHORT COMMUNICATION

Effects of delayed feeding, sodium butyrate and glutamine on intestinal permeability in newly-hatched broiler chickens

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Short title: Intestinal permeability in delayed fed chickens

Summary

The aim of the current study was to investigate the effects of delayed feeding, and supplementation with sodium butyrate or glutamine in drinking water, on intestinal permeability (IP) in young broiler chickens. Newly-hatched male chickens (n = 144, Ross 308) were allocated to four groups comprising (i) Control, (ii) 24 hours delayed fed (DF), (iii) DF supplemented with sodium butyrate (0.1%) in the drinking water and (iv) DF supplemented with glutamine (1%) in the drinking water. On days 2, 4 and 7, twelve birds per group were randomly selected, weighed and orally gavaged with fluorescein isothiocyanate dextran (FITC-d) at 2.2 mg / ml / chicken. Serum FITC-d concentration was analysed by spectrophotometry while serum diamine oxidase and D-lactic acid concentrations were analysed by microplate reader. FITC-d concentrations in the Control and DF groups were not statistically different on any day, suggesting that delayed feeding did not affect IP. Additionally, sodium butyrate increased IP compared to DF and Control on day 2 only ($p < 0.05$), while glutamine increased IP on all days, compared to DF and Control ($p < 0.05$). Diamine oxidase and D-lactic acid concentrations of all groups were not statistically different. This study suggests that a 24 hour delay in feed access during early life in chickens does not increase IP.

Key words

Fluorescein isothiocyanate dextran, Diamine oxidase, D-lactic acid, leaky gut and feed withdrawal

Introduction

Timing of the first feed in newly hatched chickens influences development of the intestinal tract and subsequent productivity. In practise, access to their first feed may be delayed by 36 – 72 hours (Noy and Uni, 2010). Geyra et al. (2001a) have shown that the intestine grows exponentially in chickens during the first 24 hours and the surface area continues to grow more slowly afterwards. Delayed feeding after hatch has been shown to affect intestinal growth by decreasing villi height (Mahmoud and Edens, 2012), crypt proliferation, enterocyte migration (Geyra et al. 2001b) and weight to length ratios of jejunum and ileum until day 4 of age (Lamot et al. 2014). Mahmoud and Edens (2012) have also shown that the adverse effects of delayed feeding continued till 14 days of age. Conversely, Shinde et al. (2014) showed that intestinal morphology and growth performance were affected by 24 hours, rather than 12 hours, post-hatch fasting. Fasting has been known to increase intestinal permeability (IP) in growing chickens (Gilani et al. 2016a). Increased IP can increase bacterial translocation and activate the immune response leading to reduced performance (Gilani et al. 2016b). Recently, chicken antibody titers against human serum albumin were shown to be raised in delayed fed chickens (Simon et al. 2015). To date, studies on increased IP in chickens have been limited and there is no published literature on whether IP is affected by delayed feeding.

Butyrate is the primary source of energy for intestinal epithelial cells and has been shown to increase villus height and crypt depth in chickens (Abdelqader and Al-Fataftah, 2016). Sodium butyrate has been utilised to improve gut health in chickens as reviewed by Ahsan et al. (2016). However, the effects of sodium butyrate on IP have yet to be investigated. Recently, sodium butyrate decreased IP in mice (Han et al. 2015) and piglets (Huang et al. 2015). Glutamine, which is a non-essential amino acid, has also been shown to decrease IP in in vitro cell cultures (Le Bacquer et al. 2003), pigs (Wang et al. 2015) and rats (Beutheu et al. 2014). However, glutamine did not improve intestinal barrier function in chickens in in vitro studies after a

mycotoxin challenge (Awad and Zentek, 2015). We have recently shown that glutamine supplementation prior to fasting challenge did not ameliorate increased IP (Gilani et al. 2017). However, its effects on improving IP after a post-hatch delayed feeding challenge have yet to be explored in chickens. The aims of this study were to investigate whether IP was increased by post-hatch delayed feeding, and also whether sodium butyrate or glutamine supplementation could ameliorate increased IP following a post-hatch delayed feeding challenge in chicks. Additionally, three biomarkers (FITC-d, D-lac, diamine oxidase) were compared for evaluating IP. These biomarkers have been utilized previously (Gilani et al. 2016a).

Materials and Methods

All procedures were approved by the Animal Ethics Committees of the University of Adelaide and the Primary Industries and Regions South Australia (PIRSA). All animal studies were performed in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Newly-hatched male chickens (n = 144, Ross 308) were obtained from a local hatchery (Baiada, Willaston, Australia). Birds were randomly allocated to four groups and kept on paper (without saw dust) with 16 h day and 8 h night cycle reared under infrared heater lamps (25 – 30 °C). The Control group had ad-libitum access to feed and water, while the remaining groups received feed and water after a delay of 24 hours. Two of the three delayed fed groups were supplemented with sodium butyrate or glutamine in water at 0.1 and 1% concentrations, respectively (Sigma Aldrich, New South Wales, Australia) for 7 days. All birds were provided commercial starter crumbles (Ridley Agri Products Pty., Murray Bridge, South Australia). Each bird was considered as a single experimental unit. On days 2, 4 and 7, chickens (n = 12 per group per day) were randomly selected, weighed and gavaged with FITC-d (4000 mol weight; Sigma Aldrich, New South Wales, Australia). FITC-d was stored at 4oC, wrapped in aluminium foil to avoid light exposure. FITC-d was gavaged at 1 ml / bird (2.2 mg FITC-d / ml of water) at three days of age. Blood was collected from the jugular vein 150 minutes after

gavage. Standards and serum samples were prepared and analysed in triplicate as previously described (Gilani et al. 2016c). Standards were spiked with FITC-d at 0, 0.0001, 0.001, 0.01, 0.1, 1.0 and 10 µg/ml to obtain a standard curve utilising a Synergy MX plate reader (Biotek Instruments, Bedfordshire, UK) at the excitation and emission wavelengths of 485 nm and 530 nm, respectively. Chicken specific antibodies of diamine oxidase and D-lactic acid Enzyme Linked Immunosorbent Assays (ELISA) were obtained from MyBioSource, (San Diego, USA).The procedure was performed as described previously (Gilani et al. 2016c). Standards and plasma samples were measured in duplicate in a microplate reader at 450 nm wavelength (Bio-Rad laboratories, California, USA) following manufacturer's instructions.

Statistical analysis

All observations on day 2, 4 and 7 were normally distributed and were included in the statistical analysis using SPSS 22 (IBM SPSS; IBM Corp., Armonk, New York, USA). Significance (p value) was measured at 0.05. Body weight and FITC-d were analysed using the general linear model (GLM) multivariate procedure and means were compared by Tamhane analysis. No significant interaction between body weight and FITC-d was found. Diamine oxidase and D-lactic acid ELISA concentrations were analysed by one way ANOVA and means were separated by Tamhane analysis.

Results

Body weights

Body weights were measured on days 2, 4 and 7 before oral gavage with FITC-d (Table 1). Body weight of the delayed fed group was significantly lower at day 2 and 4 than Control. Although the mean body weight remained lower at day 7, it was not statistically significant ($p > 0.05$). Body weights of the sodium butyrate and glutamine supplemented groups were not significantly different on each day compared with delayed fed. However, sodium butyrate

improved body weight on day 7 and was comparable to the Control ($p > 0.05$), while glutamine supplementation failed to improve body weights compared with Control on each day ($p < 0.05$).

Fluorescein isothiocyanate dextran (FITC-d), diamine oxidase (DAO) and D-lactic acid ELISA

FITC-d concentrations of the delayed fed group were not statistically different compared to Control on all days (Table 1). FITC-d concentrations within Control and delayed fed groups increased on day 7 compared to day 4 ($p < 0.05$). The concentration of FITC-d in the sodium butyrate supplemented group was significantly higher on day 2 compared to Control and the delayed fed group. Although, mean FITC-d concentrations were higher in the sodium butyrate group compared to the Control and DF groups on day 4 and 7, these were not statistically significant ($p > 0.05$). FITC-d concentrations of glutamine supplemented group were significantly higher than those of the Control and delayed fed group on all days. Additionally, FITC-d concentration increased significantly compared to the sodium butyrate supplemented group on day 7. D-lactic acid and DAO levels of all groups were not statistically different compared to Control (Table 1).

Discussion

Lower body weights in the delayed fed group up to seven days of age were comparable with previous studies (Geyra et al. 2001b, Lamot et al. 2014), while the finding that there was no significant increment in body weight in the sodium butyrate treated group compared to the DF group was contrary to the literature (Ahsan et al. 2016). In these earlier studies, delayed feeding was not investigated. Also, body weight was measured over a time period of two and four weeks compared to the current study in which body weight was measured in the first week post-hatch. However, in the current study, body weights of the sodium butyrate supplemented group were not statistically different compared to Control. This is comparable to previous studies (Ahsan

et al. 2016) which suggested that sodium butyrate demonstrated positive effects. Lower body weights in the glutamine supplemented group compared to DF alone are contrary to a previous study (Murakami et al. 2007). However, decreased body weights compared to DF and the Control are comparable to another study in which glutamine supplementation decreased body weight in chickens (Bartell and Batal, 2007). The differences in previous studies could have been due to different crude protein in the diets. Higher crude protein diets (22% in the previous and current study) with the added glutamine decreased body weight. However, in these earlier studies, delayed feeding was not investigated. Finally, glutamine supplementation could not reverse reduced body weight due to delayed feeding, consistent with the recent study in delayed-fed chickens (Zulkifli et al. 2016).

In terms of IP, no previous study has been conducted in post-hatch delayed fed chickens. Fasting in older chickens has been shown to increase IP (Gilani et al. 2016a, Gilani et al. 2017). The current study is the first to report that delayed feeding did not increase IP as indicated by FITC-d concentrations on day 2, 4 and 7. There are two potential explanations for this. Firstly, delayed feeding may not have increased IP in very young chickens, possibly due to some yolk being absorbed into the small intestine (Noy and Sklan, 2001), potentially reducing the effect of fasting on IP. Secondly, it is also possible that feeding after 24 hours restored the permeability to a healthy state. In an earlier study it was shown that microscopic changes in the enterocytes of fasted chickens were reversed after refeeding for 24 hours (Yamauchi and Tarachai, 2000). However, IP was not measured in that study. Nonetheless, the current study shows for the first time that 24 – hour delayed feeding did not increase IP on days 2, 4 and 7. Additionally, the current study has shown that FITC-d permeation in Control birds on day 2 and 4 was 0.6 µg/ml, which increased to 0.8 µg/ml on day 7 ($p < 0.05$), suggesting that intestinal permeability can change quickly with age, even in normal healthy chickens. These concentrations are numerically lower than our previous studies (Gilani et al. 2016a) in which

Control birds were shown to have FITC-d permeation at 1.91 µg/ml. This could have been due to age differences in birds, 21 days of age compared to seven days of age in the current study. Vicuna et al. (2015) have shown FITC-d concentration of 0.2 µg/ml in young chickens. Finally, when compared to the Control and DF groups, supplementation with glutamine increased IP on each day ($p < 0.05$), whereas supplementation with sodium butyrate increased IP on day 2 ($p < 0.05$) and on day 4 and 7 only numerically ($p > 0.05$). These results are incongruent with previous studies conducted in other species (Beutheu et al. 2014, Han et al. 2015, Huang et al. 2015, Le Bacquer et al. 2003). However, this is the first time the effects of these additives on IP have been investigated in newly-hatched chickens in vivo, and require further investigation. Possible reasons why these feed additives showed different results are summarised as follows. Sodium butyrate has been shown to increase IP in rat colon in Ussing chambers (Mariadason et al. 1999). In a recent study it was reported that over production of short chain fatty acids led to increased IP in rats (Ten Bruggencate et al. 2005). Additionally, in another study, sodium butyrate decreased IP at 2 mM concentration and to increase IP at 8 mM concentration in human Caco-2 cells (Peng et al. 2007). This suggests that the effects of sodium butyrate on IP may be dose dependent and hence further research is required to find the optimal dose for improving IP in chickens.

The European Food Safety Authority (EFSA) has concluded in their report of scientific opinion that there was insufficient evidence for glutamine in improving IP in humans. (EFSA 2011). In a recent study in humans, mortality was increased in a glutamine supplemented group in critically ill patients (Mundi et al. 2016). Similarly, IP was not different in glutamine supplemented subjects in Crohn's disease (Den Hond et al. 1999) and in children following digestive tract surgery (Albers et al. 2005). These studies, along with the limited literature in chickens, suggest that further investigations are required for utilising glutamine to improve IP in chickens. Potentially, glutamine could be converted to nitric oxide (Santos et al. 2014) which

has been known to increase intestinal damage at higher concentrations (Mercer et al. 1996). Since glutamine was supplemented through water in addition to the normal diet, the excess nitrogen may have caused detrimental effects on IP in chickens as reflected in the body weight results in the current study.

DAO and D-lactic acid tests have been utilised in chickens as biomarkers of increased IP (Gilani et al. 2016b). The results, however, were not different significantly and the probable reason could be that sodium butyrate or glutamine at the tested levels did not cause severe intestinal inflammation. This may have led to FITC-d permeation but not permeation of DAO and D-lactic acid, as the molecular weights of DAO and D-lactic acid are much higher (250 k Dalton(Da)) compared to FITC-d (4 k Da) (Gilani et al. 2016b). We conclude that delayed feeding did not impact on IP as measured on days 2, 4 and 7 of age in chickens. Additionally, sodium butyrate and glutamine increased IP in vivo. Further studies are required to investigate the optimal dose of sodium butyrate and glutamine for their potential to improve IP in chickens.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Table 1 Body weights (BW), fluorescein isothiocyanate dextran (FITC-d), diamine oxidase (DAO) and D-lactic acid concentrations in serum of newly-hatched chickens measured on days 2, 4, and 7

Treatments	N	Control	Delayed Fed (DF)	DF + Sodium butyrate	DF + Glutamine	S.E.M	P values					
							Con v. DF	Con v.DF+SB	Con v. DF+G	DF v.DF+SB	DF v.DF+G	DF+SB v. DF+G
BW at day 2	12	66.8 ^{a}	60.0 ^{b}	61.2 ^{ab}	56.9 ^{b}	0.17	0.01	0.09	0.00	0.94	0.48	0.20
BW at day 4	12	110.6 ^{a}	97.5 ^{b}	96.5 ^{b}	94.0 ^{b}	0.93	0.01	0.01	0.00	0.99	0.69	0.86
BW at day 7	12	186.2 ^{a}	163.9 ^{ab}	179.9 ^{a}	148.9 ^{b}	1.46	0.09	0.89	0.00	0.30	0.35	0.01
FITC-d at day 2	12	0.62 ^{a}	0.68 ^{a}	1.20 ^{b}	1.28 ^{b}	0.05	0.99	0.00	0.00	0.00	0.00	0.34
FITC-d at day 4	12	0.58 ^{aA}	0.59 ^{aA}	0.76 ^{ac}	0.73 ^{bc}	0.02	1.00	0.08	0.01	0.11	0.02	0.89
FITC-d at day 7	12	0.78 ^{aB}	0.79 ^{aB}	0.86 ^{a}	1.06 ^{b}	0.03	1.00	0.21	0.00	0.35	0.00	0.02
DAO at day 4	10	0.39 ^{a}	0.44 ^{a}	0.40 ^{a}	0.40 ^{a}	0.02	0.86	1.00	1.00	0.91	0.91	1.00
D-lactic acid at day 7	10	6.28 ^{a}	5.70 ^{a}	5.03 ^{a}	5.23 ^{a}	0.12	0.73	0.13	0.25	0.61	0.82	0.98

Means with the same superscript within a row are not significantly different ($p > 0.05$). S.E.M is standard error mean. Con is control, DF is delayed fed, SB is sodium butyrate and G is glutamine.

^{A,B} Values within a column with different superscripts differ significantly ($p < 0.05$).

Chapter 7: Gene expression and morphological changes in the intestinal mucosa associated with increased permeability induced by short-term fasting in chickens

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Name of Principal Author (Candidate)	**Syed Saad Akhtar Hussain Gilani**		
Contribution to the Paper	**Performed literature search, planned and conducted the experiment, analysed the blood samples and data, wrote the manuscript and acted as corresponding author**		
Overall percentage (%)	**70**		
Certification:	**This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.**		
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By signing the Statement of Authorship, each author certifies that:

i. **the candidate's stated contribution to the publication is accurate (as detailed above);**
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ORIGINAL RESEARCH PAPER

Gene expression and morphological changes in the intestinal mucosa associated with increased permeability induced by short-term fasting in chickens

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Abstract

Short-term fasting for 4.5 and 9 hours has been demonstrated to increase intestinal permeability (IP) in chickens. This study aimed to investigate the effects of 0, 4.5, 9 and 19.5 hours fasting on intestinal gene expression and villus-crypt architecture of enterocytes in jejunal and ileal samples. On day 38, Ross-308 male birds were fasted according to their group and then euthanized. Two separate intestinal sections (each 2 cm long, jejunum and ileum) were collected. One section was utilised for villus height and crypt depth measurements. The second section was snap-frozen in liquid nitrogen for quantitative polymerase chain reaction (qPCR) analysis of tight junction proteins (TJP) including claudin-1, claudin-3, occludin, zonula occludens (ZO-1, ZO-2), junctional adhesion molecules (JAM), E-cadherin. Additionally genes involved in enterocyte protection including glucagon like peptide (GLP-2), heat shock protein (HSP-70), intestinal alkaline phosphatase (IAP), mammalian target of rapamycin (mTOR), toll like receptors (TLR-4), mucin (MUC-2), cluster differentiation (CD-36) and fatty acid binding protein (FABP-6) were also analysed. Normally distributed data were analysed using one way ANOVA. Other data were analysed by non-parametric one way ANOVA. Villus height and crypt depth were increased ($p < 0.05$) only in the ileum after fasting for 4.5 and 9 hours compared with non-fasting group. mRNA expression of claudin-3 was significantly reduced in the ileum of birds fasted for 9 and 19.5 hours, suggesting a role in IP modulation. However, all other TJP genes examined were not statistically different from control. Nevertheless, ileal FABP-6 of all fasted groups was significantly reduced, which could possibly be due to reduced bile acid production during fasting.

Key words; Tight junction protein, intestinal permeability, reduced fasting, broiler, leaky gut

Introduction

Enterocyte structure has been extensively characterised and described in chickens (Uni et al., 1998). Enterocytes are highly cohesive, achieved in part through a network of proteins known as tight junction proteins (TJP) which include claudins, occludin, junctional adhesive molecules (JAM) and zonula occludens (ZO). These TJP regulate paracellular permeability. When TJP are disrupted, intestinal contents pass between enterocytes. This is referred to as increased intestinal permeability (IP). Increased IP can lead to increased bacterial and toxin translocation and decrease performance and health status of animals as reviewed by Gilani et al. (2016a). Long-term fasting has been demonstrated to negatively affect the intestinal morphology in chickens (Yamauchi et al., 1995 and 1996; Thompson and Applegate 2006) and recently, has been shown to increase intestinal permeability (Gilani et al., 2016b; Kuttappan et al., 2015; Vicuna et al., 2015). Gilani et al. (2017) have shown that fasting for as little as 4.5 and 9 hours increased IP in chickens. In these studies, increased IP was measured by fluorescein isothiocyanate dextran (FITC-d) or lactulose rhamnose and mannitol (LMR) sugars, which pass through tight junctions between enterocytes (Gilani et al., 2016a). Fasting prior to slaughter is often applied in the chicken meat industry to avoid contamination of the intestinal contents with the edible parts, and increased IP may result in increased bacterial translocation into edible tissue. Therefore it is necessary to investigate the effects of fasting on IP in order to determine whether intestinal morphology and IP are linked. Although, there have been no direct studies in chickens, studies conducted in rat and human models have reported a mechanism underlying fasting and increased IP. In rats, fasting led to reduced mRNA expressions of claudin, occludin and zonula occludens (Hamarneh et al., 2014) and fasting for three days led to the death of enterocytes (Iwakiri et al., 2001). Nutrient starvation of *in vitro* cell lines (Caco-2) led to increased IP measured by trans-epithelial electrical resistance due to reduced claudin-2 protein

formation (Nighot et al., 2015). These studies suggest that TJP may also be involved in increasing IP in chickens.

In addition to the altered expression of TJP, differential expression of many genes involved in the protection and inflammation of enterocytes may explain the mechanism of fasting- induced IP. These genes included Heat shock protein (HSP-70), Glucagon like peptide (GLP-2), Mammalian target of rapamycin (mTOR), Sodium dependent glucose transporter (SGLT-1), Intestinal alkaline phosphatase (IAP), Cluster differentiation (CD-36), TLR-4 (toll like receptor-4), Mucin and fatty acid binding protein (FABP-6).

Since fasting periods of 4.5, 9 and 19.5 hours have been shown to increase IP in chickens (Gilani et al., 2017), the objectives of this study were to investigate the associated effects of fasting of chickens for 0, 4.5, 9 and 19.5 hours on mRNA expression of TJP including claudin-1, claudin-3, occludin, JAM, E-cadherin, ZO-1, ZO-2 and expression of genes associated with cellular protection and inflammation such as HSP-70, IAP, GLP-2, mTOR, FABP-6 and MUC-2, and villus-crypt architecture of enterocytes.

Materials and Methods

Experimental protocols

All protocols were approved by the Animal Ethics Committees of the University of Adelaide and the Primary Industries and Regions South Australia (PIRSA). All animal studies were performed in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Animals, diets, tissues sampling and processing

Jejunum and ileum samples were obtained from the study reported by Gilani et al. (2017). Briefly, Ross-308 male chickens (n=48) were reared and fed starter, grower and finisher diets

during 0 – 9, 10 – 23 and 24 – 38 days of age, respectively, to meet the Ross-308 nutrient requirements (Ross, 2014) (Table 1). On day 35, all birds were transferred to metabolism group cages (two birds per cage). After two days of adaptation, chickens were transferred to individual cages and distributed to four treatments; 0, 4.5, 9 and 19.5 hours fasting (n = 12 per treatment group). Following their respective fasting periods, birds were euthanized by cervical dislocation and intestinal sections were collected. Two separate sections (2 cm each) of jejunum and ileum from the same bird were sampled from six birds per treatment group (n = 6) for analysis. All sections were washed with ice cold phosphate buffer solution (Sigma Aldrich, New South Wales, Australia). One section was snap-frozen in liquid nitrogen and then stored at -80^{0}C until mRNA expression analysis. The second section was retained for histological analysis as described previously (Forder et al., 2007). Briefly, second section was opened and placed on a small piece of blotting paper and stored in 10% buffered formalin for histological analysis. After fixation, samples were embedded in paraffin wax and stained with hematoxylin and eosin (H&E). At least 10 measurements (150 µm apart) were taken from each section and in total 6 birds were measured from each group. Villus height (µm), crypt depth (µm) and villus area (µm^2) were measured using an Olympus digital camera and Video Pro 32 imaging software (Leading Edge Pty Ltd). Quantitative polymerase chain reaction (qPCR) processes including isolation and quantification were performed according to Forder et al. (2012) and Kitessa et al. (2014) as described in detail below.

Total RNA isolation and quantification

RNA isolation was performed using a commercial kit (RNeasy Mini kit, Qiagen, Hilden, Germany). Samples frozen in liquid nitrogen were wrapped in alfoil and fragmented by a hammer. Approximately 100 mg of each sample was weighed, mixed with 2 ml of Trizol reagent (Invitrogen, Carlsbad, USA) and homogenized immediately using an Ultra-Turrax (T25; IKA-Werke GmbH and Co. KG, Staufen, Germany). Tubes were centrifuged at 10,000

g (Mikro200; Andreas Hettich GmbH and Co. KG, Tuttlingen, Germany) for 15 min at 4°C and the upper aqueous phase was collected for further use. This solution (300 µL) was mixed with an equal volume of 70% Ethyl alcohol (Sigma-Aldrich, St. Louis, U.S.A) and loaded onto RNeasy mini columns, and centrifuged at 8,000 × g for 1 min at room temperature (Mikro200; Andreas Hettich GmbH and Co. KG, Tuttlingen, Germany). Subsequent purification steps were performed according to the manufacturer's instructions with the total RNA eluted in 50ul EB buffer. Ultra violet spectrophotometry (Nanodrop 2000; Thermo Scientific, Wilmington, DE) was utilized to determine the concentration and purity of total RNA. Agarose gel electrophoresis was used to confirm the integrity of the total RNA in all samples.

Design of quantitative PCR (qPCR) assays and reverse transcription of complementary DNA (cDNA)

The chicken genome sequence (http://www.ensembl.org/Gallus_gallus ; August 2016 version 85) was used to design oligonucleotides for the qPCR assays shown in Table 2. In general, the amplicon size of the qPCR assays was less than 100bp, and oligonucleotide pairs were selected on the basis that they flanked introns greater than 500bp in length. For cDNA synthesis, the RNA concentrations of all samples were standardized to 200 ng/µl using liquid-handling robotics system (EpMotion 5075; Eppendorf, Hamburg, Germany). One microgram of total RNA was converted to cDNA using random hexamers and the High Capacity cDNA synthesis kit (Applied Biosystems, Carsbad, USA). The cDNA synthesis reactions were performed at 39 °C for 2 hours and the reverse transcriptase was subsequently inactivated at 65°C for 20 minutes. cDNA was diluted 1:4 with 10mM Tris (pH 8.0; Ambion, Austin, USA) and stored until required at -80 °C.

Validation of qPCR assay specificity and performance and assessment of mRNA levels

A portion of cDNA from each sample was pooled to generate a single sample that was representative of the entire study. Seven consecutive 2-fold serial dilutions were performed on the pooled cDNA (1:4) to generate a standard curve (1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512) which was used to assess the reaction efficiency and specificity of each of the qPCR assays. The qPCR assays (300nM) were measured in triplicate against the cDNA standard curve on a 384 well real-time PCR machine (7900; Applied Biosystems) using PowerSYBR (10ul; Applied Biosystems, USA) and the following cycling parameters: 95°C/10 min for 1 cycle, and 95°C/15 s and 60°C/1 min for 40 cycles, with data acquisition occurring at the 60°C step. All assays that produced a single amplification product and had a reaction efficiency exceeding 90% were included in the study. The assessment of mRNA levels was performed in triplicate as described above using the same standard curve whilst the cDNA samples were diluted to1:20 with Tris-HCl (pH 8.0 Ambion, USA). At the completion of each qPCR run the cycle threshold was manually adjusted to 0.3 and text files were exported from the SDS2.3 software (Applied Biosystems, USA).

Processing and normalization of real time PCR data

Data were processed using SDS 2.3 software (Applied Biosystems) Reference genes for data normalization, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and TATA-binding protein (TBP), were selected because they were stably expressed in chicken intestine (Forder et al., 2012; Kitessa et al., 2014). The stability of the GAPDH, TBP and all other genes measured in this study was assessed via a NormFinder (Andersen et al., 2004) analysis using the Genex software package (MultiD, Göteborg, Sweden).

Statistical analysis

All statistical analyses were performed using SAS 9.4 (SAS, Inc., NC: USA), and results were compared using one-way analysis of variance (ANOVA) except for FABP-6 and CD-36 for which non-parametric ANOVA was utilized. For gene expression analysis, the data for jejunal claudin-3, ZO-2, HSP-70, MUC-2, IAP and ileal ZO-2, JAM-2, HSP-70, SGLT-1, TLR-4 and IAP were square root transformed to fulfil the equal variance condition of ANOVA. Normality was measured using univariate normal plots and statistical significance was accepted when $p < 0.05$. One outlier (mean $\pm$ 3 standard deviations) from each of three treatment groups was excluded. These groups were non-fasting chickens (ileum JAM-2), 4.5 hours (jejunum HSP-70) and 9 hours (jejunum FABP-6). All data presented in Table 4 are the untransformed relative mRNA levels of each gene. They represent the fold increase/decrease in mRNA level of each gene in relation to the reference gene (TBP).

Results

Villus height, crypt depth and villus area

Villus height in the jejunum was not significantly affected by fasting time (Table 3). However, villus height in the ileum was increased significantly ($p < 0.05$) in the 4.5 and 9 hours fasting compared with non-fasting group. Although villus height was also numerically higher in 19.5 the hours fasting group in the ileum, the difference was not statistically significant. Crypt depth in the jejunum was increased ($p < 0.05$) only in the 4.5 hours fasted birds compared with non-fasting group. Crypt depth in the ileum increased significantly for 4.5 and 9 hours fasting compared to non-fasting. Villus area in the jejunum increased significantly for 9 and 19.5 hours fasting, while villus area in the ileum of all groups was not significantly different.

mRNA expression of TJP and other genes

Tight junction protein expression of claudin-3 was significantly reduced in the ileum for 9 and 19.5 hours compared to non-fasting group. However, other genes including E-cadherin, JAM-2, ZO-1, ZO-2, claudin-1 and occludin were not significantly different from non-fastings in the jejunum and the ileum (Table 4). Gene expression of FABP-6 was significantly reduced in the ileum only for each fasted group compared to non-fastings. All other genes were not significantly different to control.

Discussion

The effect of fasting on the intestinal morphology in chickens has been extensively studied (Yamauchi and Tarachai, 2000; Yamauchi *et al.*, 1995 and 1996). However, these studies involved fasting for more than 24 hours and the focus was not on increased IP. Increased villus height increases nutrient absorption and increased crypt depth indicates increased proliferation (Thompson and Applegate 2006). In the current study, increased ileal villus height in birds fasted for 4.5 and 9 hours suggested that birds could adjust their nutrient absorption when it is lacking. Similarly, increased ileal crypt depth in birds fasted for 4.5 and 9 hours suggested that new enterocytes were being formed. However, unaltered villus height and crypt depth in the ileum for 19.5 hours fasting suggested that the intestine could recover from the initial shock of food deprivation.

One aim of this study was to investigate whether intestinal morphology can be associated with increased IP. However, villus height in the ileum increased after 4.5 and 9 hours fasting and decreased inthe19.5 hours fasted group. Villus height in the jejunum was not changed. Interestingly, Gilani et al. (2017) have shown that 4.5, 9 and 19.5 hours fasting increased IP linearly, as measured by increased serum FITC-d concentrations in blood, suggesting that the

villus morphology in the current study did not directly correlate with the increased IP. This phenomenon has been observed in other studies. Increased IP has been observed in association with increased villus height and crypt depth in pigs (Yeruva et al., 2016), decreased villus height and crypt depth in pigs (Spreeuwenberg et al., 2001) and unaltered intestinal morphology in rats (Hodin et al., 2011) and humans (Hernandez et al., 1999). Furthermore, reduced villus area in the jejunum was not correlated with thio-urea absorption after three days of fasting in chickens (Levin and Mitchell, 1984). This suggests that intestinal morphology may not have a direct influence on IP.

This is the first study to investigate mRNA expression of TJP in chickens following short -term fasting-induced IP. The NormFinder analysis revealed that TBP was the most stably expressed gene measured in the study followed closely by mTOR. Unlike the previous studies (Forder et al., 2012; Kitessa et al., 2014) GAPDH was not a suitable candidate for data normalization, whereas TBP was ideally suited to this purpose. The results of the current study revealed that ileal mRNA expression of only claudin-3 was significantly reduced after 9 and 19.5 hours fasting compared to control. It is possible that while 4.5 hours fasting was sufficient to increase IP, it may have been too short to show a significant reduction in mRNA expression, whereas greater fasting periods did change gene expression. This suggested that claudin-3 may have been involved in fasting-induced IP. However, other TJP genes did not show any significant changes. It is also possible that some TJP reassemble during the challenge making it difficult to study only a few genes as suggested by a mycotoxin challenge in chickens (Osselaere et al., 2013). In the aforementioned study, claudin-5 was significantly increased, whereas claudin-1, ZO-1 and ZO-2 were not affected. Further studies with additional TJP genes (ZO-3, claudin-2, claudin-4, claudin-5) may help to better understand the mechanism.

Unaltered TJP expression in the jejunum and the ileum ($p > 0.05$) was unexpected. Gilani et al. (2017) have shown that these fasting periods increased IP, as measured by FITC-d.

Alsothese results were inconsistent with the studies in other species. In human studies, food deprivation led to decreased ZO-1, occludin, E-cadherin and claudin-4 (Ralls et al., 2015). Fasting has also been reported to reduce claudin-2 expression after 6 and 24 hours food deprivation in human subjects measured by western blotting (Nighot et al., 2015). Conversely, fasting in piglets did not alter occludin, ZO-1 and claudin genes significantly (Horn et al., 2014), however water restriction reduced the mRNA expression of these genes. Since all birds in the current study were provided *ad-libitum* water, this suggested that water restriction together with fasting may have had synergistic effects in chickens, which is worthy of further study. In another study in chickens, mRNA expression of ZO-1, JAM-2, JAM-3, claudin-1 and occludin were not significantly different compared to control, while intestinal permeability was increased as measured by serum endotoxin following a coccidiosis challenge (Chen et al., 2015). Coccidiosis challenge severely damages the gut and may increase mortality and morbidity compared with fasting and implies that IP changes may not always be related to changes in the expression of TJP genes. A similar mechanism has been reported in which significant changes in TJP in immunohistochemistry were not translated into changes in mRNA of TJP in a chemotherapy-induced gut toxicity rat model (Wardill et al., 2014). Furthermore, Wardill et al. (2014) suggested that posttranslational changes may have contributed to this observation. Posttranslational changes modify protein structure during synthesis which has been shown in occludin and claudin (Cummins, 2012). TJP are highly dynamic and their prompt remodelling has also been suggested (Capaldo and Nusrat, 2015).This may explain why TJP expression was unaltered by fasting in the current study.

In order to understand whether any protective mechanisms were involved, additional genes were studied. HSP-70 is produced in response to oxidative stress and has a protective role for gastrointestinal cells against intestinal inflammation and IP changes (Arnal and Lalles 2016; deFoneska et al., 2010; Weitzel and Wischmeyer 2010). HSP-70 expression was not altered in

the jejunum and ileum after fasting, similar to another study in which fasting did not change expression of this gene in the liver (Delezie et a., 2007).Glucagon like peptide (GLP-2) produced in the intestine, has been shown to reduce intestinal inflammation (Burrin et al., 2003; Richards and McMurtry 2009), permeability (Dong et al., 2014) and bacterial translocation (Burrin et al., 2003; Said et al., 2015). GLP-2 results are comparable to another study in chickens, in which fasting did not alter GLP-2 expression in the proventriculus and the duodenum (Richards and McMurtry, 2009). Intestinal alkaline phosphatase (IAP) is produced in the intestine. It digests phosphate in the diet and is considered important for reducing intestinal inflammation, as reviewed by Melo et al., (2016). In a human study, mRNA expression of IAP was significantly reduced due to fasting and was correlated with damage to the TJP (Hamarneh et al., 2014). IAP expression in the fasting treatments were also not altered. Lack of significant decrease in HSP-70, GLP-2 and IAP in the jejunum and the ileum suggest that these are not associated with increased IP in chickens following a fasting challenge.

Prolonged fasting has recently been suggested to be involved in intestinal inflammation because of reduced serum antioxidant levels (Abdeen et al., 2009) and increased immunoglobulin (Lara-Padilla et al., 2011) in humans. Cluster differentiation (CD-36) has been involved in fatty acid transportation (Kitessa et al., 2014), although recently it has been associated with binding with a pro-inflammatory cytokine, TLR-4 (toll like receptor-4) (Abumrad and Goldberg 2016). TLR-4 binds with the bacterial toxins and has been involved in gut inflammation (Andrade et al., 2015). CD-36 along with TLR-4 may therefore explain increased IP in chickens However, both genes did not show significant changes in the jejunum and ileum suggesting no involvement of CD-36 in TLR4 activation. Mammalian target of rapamycin (mTOR) is a signalling pathway protein and has been shown to modulate cell growth, proliferation (Kechen and Kozar 2012) and recently has been involved in the GLP-2 pathway to regulate IP in *in vitro* studies (Yu et al., 2016).Unaltered mTOR expression results

in the jejunum and ileum are in agreement with another study in chickens (Hu et al., 2016) and suggest this gene was not related to gut protection in the fasting model. Mucin is considered as the first line of defence against any pathological and inflammation of the gut (Forder et al., 2012). However, its role in fasting induced IP in chickens has not been studied. Unaltered expressions of MUC-2 suggested that inflammation may not have been involved in fasting and this may have been due to duration of fasting (short versus three days of fasting in the aforementioned study). However, other inflammatory genes such as interleukin-8 should also be considered in future studies. Fatty acid binding protein (FABP-6) has been known for its role in fatty acids transport. Chen et al. (2015) suggested recently that FABP-6 was involved in IP in chickens. Sodium dependent glucose transporter (SGLT-1) has been implicated in reduced IP in pigs induced by LPS (Yu et al., 2005) and increased SGLT-1 was also observed in heat stress induced IP in pigs (Pearce et al., 2013). However, this gene was also not altered suggesting this is not involved in the gut protection. FABP-6 is expressed in the ileum, binds bile acids and transports fatty acids across the epithelium (Smathers and Petersen, 2011). However, its overexpression has been linked with colorectal cancer in humans and damage to the intestinal epithelium (Ohmachi et al., 2006). FABP-6 was decreased in the large intestine of mice (Iseki et al., 1989), while FABP (variant not specified) was unaltered in the chickens fasted for 24 hours (Shinde et al., 2014). However, IP was not measured in these studies and different variants of genes may have a different role. FABP-6 in the jejunum was increased significantly in chickens, with the increased IP measured by increased serum endotoxins (Chen et al., 2015). These contradictory studies imply that further research is needed to understand FABP-6 involvement in IP. Meanwhile, reduced FABP-6 in the ileum could be explained by other findings in which FABP-6 was linked with reduced bile acid production due to fasting (Smathers and Petersen 2011).

Conclusions

Our results suggested that TJP claudin-3 was disrupted as a consequence of fasting. However, further studies with additional genes and their variants may advance our understanding of the mechanism of fasting induced increased IP. Once established, this mechanism could be modulated to avoid TJP disruption during fasting in chicken meat production, or during period of stress when feed intake is severely reduced.

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Table 1 Composition of diets

Ingredients %	Starter	Grower	Finisher
Wheat	32.54	36.65	41.90
Soybean meal	31.72	26.71	22.99
Sorghum	20.00	19.99	20.00
Canola meal expeller	5.00	6.02	4.78
Canola oil	4.98	4.99	5.35
Di-calcium phosphate	1.78	1.55	1.35
Limestone	1.17	1.09	1.01
L- Alanine	1.22	1.22	1.22
Sodium bicarbonate	0.39	0.38	0.36
DL-Methionine	0.35	0.31	0.26
L- Lysine	0.29	0.27	0.22
L-Threonine	0.17	0.17	0.12
Premix + Xylanase	0.15	0.15	0.15
Salt	0.14	0.14	0.15
Zinc Oxide	0.015	0.015	0.015
Choline Chloride	0.062	0.076	0.082
Sand	0.005	0.21	0.005
Calculated nutrients % (unless otherwise specified)			
Dry matter	88.8	88.7	88.4
ME MJ/kg	12.6	13.0	13.0
Crude protein	22.6	20.7	19.5
Crude fat	6.8	7.9	7.2
Crude fibre	2.6	2.6	2.6
Ash	5.9	5.6	4.9
Digestible lysine	1.3	1.2	1.0
Digestible methionine	0.7	0.6	0.5
Digestible cysteine	0.3	0.3	0.3
Digestible threonine	0.8	0.8	0.7
Digestible tryptophan	0.3	0.2	0.2
Calcium	0.9	0.9	0.8
Phosphorus available	0.5	0.4	0.4

Composition of the premix per kg of diet; vit. A 14000 IU, vit. D_3 5000 IU, vit. E 75 mg, vit. K_3 3.75mg, vit. B_1 3 mg, vit. B_2 9 mg, vit. B_6 5mg, vit. B_{12} 0.03 mg, Biotin 0.2 mg, Pantothenic acid 15 mg, Folic acid 2.5 mg, Niacin 55 mg, Copper 20 mg, Cobalt 0.25 mg, Iodine 1.25 mg, Iron 40 mg, Manganese 120 mg, Molybdenum 2 mg, Selenium 0.3 mg, Phytase 100 mg , Ethoxyquin 100mg.

Table 2 Oligonucleotides used for quantitative reverse transcriptase PCR assays

Genes	Group	Forward	Reverse	Amplicon size	Accession number
Claudin-1	Tight junction protein	AAGGTGTACGACTCGCTGCT	CAGCAACAAACACACCAACC	102	ENSGALG00000026862 NP_001013629
Claudin-3		GCCAAGATCACCATCGTCTC	CACCAGCGGGTTGTAGAAAT	113	ENSGALG00000022557 NP_989533
E-cadherin		GCTGCAGAGACCTTCAGCTT	CCTTGAAGCGTGTGTCAGTC	133	ENSGALG00000000608 CGNC Symbol;Acc:377
JAM-2		AGACAGGAACAGGCAGTGCT	TCCAATCCCATTTGAGGCTA	134	ENSGALG00000015746 CGNC Symbol;Acc:11746
ZO-1		AAGTGGGAAGAATGCCAAAA	GGTCCTTGGATCCCGTATCT	133	ENSGALG00000003970 CGNC Symbol;Acc:2914
ZO-2		GCCCAGCAGATGGATTACTT	TGGCCACTTTTCCACTTTTC	120	ENSGALG00000015109 CGNC Symbol;Acc:49456
Occludin		ACGGCAAAGCCAACATCTAC	ATCCGCCACGTTCTTCAC	86	ENSGALG00000027456 CGNC Not available
TLR-4	Inflammatory genes	CTGCAGTTTCTGGATCTTTCAA	TAAGCCATGGAAGGCTGCTA	138	ENSGALG00000007001 CGNC Symbol;Acc:5282
CD-36		CATCACGTGCCTCAACCTC	TTCTGTTCTGCAGCATGTTTG	116	ENSGALG00000008439 CGNC Symbol;Acc:6399
mTOR		CTTTCCGTCCTTCAGCATTC	CTGACAGCCACAGAAAGCAA	133	ENSGALG00000003339 CGNC Symbol;Acc:2430
MUC-2		ATTGAAGCCAGCAATGGTGT	TTGTTGGCCTTGTCATCAAA	125	ENSGALG00000006744 CGNC Symbol;Acc:51889
IAP	Gut protective genes	GAGCCTACACCAGCATCCTC	GCTGCCTGTAGTCCTTGTCC	108	ENSGALG00000023866 CGNC Symbol;Acc:52291
GLP-2		CGTGCCACAGCCATTCTTA	AGCGGCTCTGCAAATGATTA	123	ENSGALG00000027187 CGNC Symbol;Acc:206
HSP-70		GGCTGGAGAGAAGAATGTGC	CAGCTGTGGACTTCACCTCA	105	ENSGALG00000011715 CGNC Symbol;Acc:51984
SGLT-1		TGCCGGAGTATCTGAGGAAG	CCCCATGGCCAACTGTATAA	140	ENSGALG00000006728 CGNC:49346
FABP-6		GAGGACGCACCACGACTAAT	TTTTCCCACCTTCCATTTTG	105	ENSGALG00000001445 CGNC Symbol;Acc:998
GAPDH	House keeping genes	CAACCCCCAATGTCTCTGTT	TCAGCAGCAGCCTTCACTAC	94	ENSGALG00000014442 CGNC Symbol;Acc:49077
TBP		GTCCACGGTGAATCTTGGTT	GCGCAGTAGTACGTGGTTCTC	128	ENSGALG00000011171 CGNC Symbol;Acc:8484

Table 3 Villus height (µm), crypt depth (µm) and villus surface area (µm^2) ± SE in jejunum and ileum (n = 6) of 0, 4.5, 9 and 19.5 fasted chickens

	Jejunum			Ileum		
Fasting	Villus height	Crypt depth	Villus area	Villus height	Crypt depth	Villus area
non-fasting	1292.2 ± 25.8 [a]	178.9 ± 5.7 [b]	190364 ± 15186 [b]	635.1 ± 21.5 [b]	143.2 ± 4.8 [b]	102584 ± 8266 [a]
4.5 hrs	1201.0 ± 27.7 [a]	207.2 ± 6.7 [a]	235019 ± 16286 [b]	817.2 ± 40.5 [a]	180.4 ± 6.5 [a]	107305 ± 8178 [a]
9.0 hrs	1328.1 ± 31.2 [a]	184.1 ± 4.7 [b]	109166 ± 7688 [a]	745.9 ± 21.3 [a]	165.2 ± 6.1 [a]	131670 ± 10059 [a]
19.5 hrs	1321.8 ± 21.4 [a]	195.5 ± 6.3 [b]	306001 ± 21017 [a]	717.3 ± 31.1 [b]	155.5 ± 4.8 [b]	81900 ± 6310 [a]

Within each column, means with different superscripts are significantly different ($p < 0.05$)

Table 4 Genes expression for 0, 4.5, 9 and 19.5 hours of fasted chickens (n = 6 unless where denoted with * where n =5)

	Jejunum						Ileum					
Genes	non-fasting	4.5 hrs fasting	9 hrs fasting	19.5 hrs fasting	S.E.M	P	non-fasting	4.5 hrs fasting	9 hrs fasting	19.5 hrs fasting	S.E.M	P
Claudin-1	1.11	0.97	1.06	1.13	0.04	0.626	1.20	1.12	1.13	1.27	0.05	0.760
Claudin-3	0.88	1.13	1.22	1.25	0.08	0.345	1.99[a]	1.54 [ab]	1.21 [b]	1.13 [b]	0.12	**0.022**
Occludin	0.94	1.04	0.94	0.95	0.04	0.782	1.02	1.17	0.85	0.98	0.05	0.172
ZO-1	1.24	1.28	1.31	1.26	0.05	0.954	1.36	1.28	1.29	1.28	0.04	0.871
ZO-2	1.54	1.90	1.62	1.52	0.1	0.578	1.85	1.48	1.57	1.48	0.06	0.126
JAM-2	1.61	1.87	1.81	1.91	0.14	0.893	2.07*	1.84	1.90	1.90	0.12	0.939
E-Cadherin	0.87	0.95	1.09	0.90	0.06	0.622	1.04	0.96	0.98	0.87	0.05	0.700
FABP-6	0.02	0.09*	0.31	0.07	0.07	0.499	22.38 [a]	7.93 [b]	3.37 [b]	2.66 [b]	1.84	**<0.001**
CD-36	4.73	4.32	2.83	6.64	0.93	0.577	1.72	6.12	2.27	2.99	0.91	0.334
HSP-70	2.56	2.98	1.99*	1.84	0.22	0.285	2.91	2.74	2.04	3.26	0.23	0.245
SGLT-1	3.12	2.90	2.99	3.576	0.31	0.903	1.83	2.61	2.55	3.00	0.28	0.551
MUC-2	2.06	2.31	2.79	1.94	0.25	0.716	2.22	1.57	2.44	2.19	0.20	0.469
TLR-4	2.81	2.70	2.98	3.03	0.18	0.927	3.34	2.73	2.71	2.86	0.14	0.480
mTOR	2.42	2.59	2.57	2.31	0.06	0.275	2.49	2.21	2.29	2.31	0.06	0.367
IAP	2.29	1.78	1.79	2.15	0.16	0.712	1.95	2.11	1.64	2.03	0.17	0.846
GLP-2	2.69	2.16	2.39	2.56	0.11	0.398	2.32	2.23	2.22	2.60	0.10	0.482

Means with different superscripts within a row and tissue type are significantly different $p < 0.05$

Chapter 8: General discussion

The basic aim of this research was to identify reliable models and biomarkers of increased intestinal permeability in chickens. Until recently, there has been a deficiency in comprehensive research conducted in chickens and this gap in knowledge prompted the current study. From the literature of other species it could be concluded that increased IP could affect performance and FCR in chickens, however, there is no literature available regarding this in chickens. As mentioned earlier any minor changes in FCR could lead to a significant effect on feed utilization and feed costs in poultry production. The detailed discussions from each chapter have been included in the relevant papers and only presented here to summarise the main findings.

This research revealed that lactulose, mannitol and rhamnose (LMR) sugars, as well as FITC-d, could be effectively utilized to evaluate IP changes in chickens. In addition, the studies in this thesis revealed that fasting could be used as a method to increase IP in chickens (Chapter 4). Although no direct relationship could be found between the sugar ratio and FITC-d methods, it was found that the FITC-d biomarker could be analysed in a relatively short time compared to the LMR sugar method. Chapter 5 also described studies which suggested that LMR sugars were more sensitive to small changes in IP compared to FITC-d. This was probably due to the smaller molecular size of lactulose (340 Daltons) compared to FITC-d (3000-5000 Daltons). At the same time, LMR sugars were deemed safe for *in vivo* use in chickens, as these sugars are utilized routinely in humans to evaluate changes in IP. It is very important to note that both of these biomarkers could be utilized in live chickens. Consequently, the birds do not need to be euthanized for IP determinations. Additionally, these biomarkers provide an opportunity to conduct further research within the same animal, before and after the initial challenge.

This project further investigated three methods of compromised barrier function, including endotoxin lipopolysaccharide, dextran sodium sulphate and fasting. LPS was injected at 0.5 and 1 mg/kg body weight, while DSS was provided in drinking water at 0.75% in these studies. Since LPS and DSS did not increase IP in chickens at the doses tested (Chapter 3 and 4), further research may be needed to investigate the optimal doses of these agents for increasing IP in chickens. Additionally, it is also possible that a different route of administration (oral gavage versus drinking water and in-feed versus intraperitoneal) of these agents could have a differential effect on IP increment. In addition, different strains of chickens, such as Ross and Cobb, could respond differently to administration of these agents. Studies in this thesis utilized the Ross strain, while Cobb were utilized in some other studies (Vicuna et al., 2015). Until recently, fasting as a method to induce IP has not been investigated in animals, with a single recent paper reporting that fasting for 24 hours increased IP in chickens (Vicuna et al., 2015). Additionally, in the LPS studies of the current thesis, fasting for 19.5 hours was applied since it was believed that fasting would inhibit the results of increased IP. In the next experiment (Chapter 4), fasting for 19.5 hours increased IP significantly, confirming the aforementioned hypothesis. Chapter 3, also showed that fasting along with LPS did not impact on IP. There was some variation in the results, which could be further explored. However, due to the fact that LPS at two doses did not impact IP, it was not further investigated. Contrarily, fasting is sometimes unavoidable in the chicken meat industry, especially when newly hatched chicks are introduced to the farm and when birds are sent to the abattoir. Additionally, fasting is routinely applied in broiler breeders for weight management and reproductive performance. Due to the reasons mentioned above, fasting was further investigated, along with the feed additives glutamine and sodium butyrate, in an attempt to ameliorate increased IP. It was further revealed that fasting for as little as 4.5 hours prior to slaughter increased IP (Chapter 5). However, glutamine, a conditionally non-essential amino acid, failed to ameliorate

increased IP when fed at 1%, prior to fasting challenge. Further research is required to investigate the optimal withholding time of feed prior to slaughter to avoid increased IP and prevent unwanted passage of intestinal contents, with subsequent contamination of human food. Once established, this may enable the chicken meat industry to reduce bacterial translocation from the gut to the organs and meat, leading to improved product safety.

The effect of delayed feeding for a maximum of 24 hours in newly hatched chickens was also investigated (Chapter 6) revealing that delayed feeding did not increase IP at this age. This may be useful for the industry to ensure that chickens are not at risk of harmful pathogens passing from the intestine to the systemic circulation following fasting for 24 hours. Alternatively, this could be further investigated to determine the maximum time of delayed feeding during which IP is not increased. However, transportation and environmental stress were not studied in this experiment. These stressors, along with delayed feeding, may impact IP differently, and should also be studied together in future studies. Finally, the potential mechanism for fasting induced increased IP in chickens was also investigated (Chapter 7). This will require further research as discussed in the following section on future directions.

Future directions

Following the failure of LPS and DSS to increase IP, fasting as a model was subsequently utilized in further studies conducted in this project. Fasting for 4.5, 9 and 19.5 hours increased IP. However, all birds were given water *ad-libitum* (except in the study for newly hatched chickens). Water deprivation may have had a confounding effect with feed restriction and further research on this aspect is required. This has been shown in pigs where fasting alone did not alter tight junction protein expression, unlike water restriction (Horn et al., 2014).

As mentioned earlier, fasting is also applied in breeders for weight management and reproduction performance, however, its effects on IP in these birds have not been investigated. There is also a possibility that increased IP could lead to an activated immune response in young chickens produced by these breeders through epigenetic effects. This is worth investigating in future investigations. Since increased IP can lead to bacterial translocation, future studies may investigate the type of pathogen being translocated or immune evoking antigens (such as LPS). Currently, there is little literature regarding bacterial translocation due to increased IP in chickens. Additionally, how increased IP can impact on FCR, daily live weight gain and disease resistance should further be investigated. Due to the limited knowledge regarding models and biomarkers of increased IP in chickens, experiments with the LPS, DSS and fasting methods, along with the different biomarkers, have been reported in this thesis. This new information will assist researchers to develop reliable models of IP in chickens in order to design more effective interventions. Future models that focus on poultry diseases (subclinical necrotic enteritis or coccidiosis, for example) or agents that induce intestinal inflammation (such as dexamethasone and indomethacin) will further enhance our knowledge of increased IP in severe stress situations.

This project has introduced new biomarkers such as diamine oxidase, d-lactate, faecal anti-trypsin inhibitor and intestinal fatty acid binding proteins for evaluating increased IP. The effects of these biomarkers in the fasting model were not significantly different than control. However, these may deliver different results in severe intestinal stress models, such as coccidiosis (Zhang et al., 2016) and mycotoxin challenges (Chen et al., 2016) in chickens. Once these biomarkers are established, they could be effectively utilized to evaluate increased IP without the necessity for giving oral gavages, as are required in the LMR sugar or FITC-d biomarker methods.

The studies involving FITC-d in the current research, and other research mentioned in this thesis, utilized similar sized FITC-d of 3000-5000 Dalton molecular size. Similar molecular sizes of FITC-d were selected to better compare published research results with the current project. Future studies, however, with different molecular sizes of FITC-d (up to 10k Dalton), could be utilized to assess varying degrees of intestinal damage (permeability). For instance, high molecular weight FITC-d molecules may only pass through disrupted TJP in chickens but be prevented from passing through TJP in healthy birds. This information could further be utilized for benchmarking IP in healthy and morbid chickens.

Additionally, the molecular mechanism of fasting-induced IP was investigated. Out of many TJPs, only claudin-3 was down-regulated following 9 and 19.5 hours fasting. Also, genes involved in protection of intestinal cells were not affected. Since fasting for as little as 4 hours increased IP as measured by FITC-d, further TJP genes and their different variants should be considered in future studies. Further research should include TJP genes such as claudin-5 and ZO-3 since different variants of these genes may impact differently. Additionally, advanced techniques such as proteomics may help to understand the mechanism of fasting-induced increased IP thorough investigation of all TJP.

Future studies should also focus on the nutrient requirement alterations during increased IP when birds are at disease risk or have an activated immune system. Following mycotoxin challenge, protein requirements of birds have been reported to be increased (Chen et al., 2016). The candidate has recently liaised with researchers of the South Australian Research and Development Institute to investigate the role of low protein diets in increased IP in chickens. This project is in its very early stages and will likely be beneficial for the poultry industry.

Finally, the role of different nutrients and different feed additives to increase intestinal barrier function (reduce leaky gut) and improve health and performance of birds should be investigated

by utilising LMR sugars and FITC-d. In this thesis, feed additives such as glutamine and sodium butyrate were also utilized in two experiments in an attempt to ameliorate fasting-induced IP. However, no effective remediation of increased IP was evident in the fasting induced IP model in chickens. This may have been related to the doses used. Additionally, as discussed previously, route of administration and chicken strain (Cobb vs Ross) may also have influenced the utilization of these feed additives. Further research is therefore needed to determine the optimal doses of glutamine and sodium butyrate in order to utilize these feed additives effectively. Finally, additional nutrients and feed additives that could be investigated utilizing the IP models and biomarkers described above include (but are not limited to) fibre (soluble and insoluble), resistant starch, different types of fatty acids (saturated, polyunsatured and branched chain fatty acids), essential oils (cinnamaldehyde and thymol), herbs (oregano, thyme, garlic), probiotics and enzymes (including phytase, non-starch polysaccharide degrading enzymes, lipase and protease).

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